Final Report
Colorado Plateau Cooperative Ecosystem Studies Unit
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Park: FLAG (WUPA)

Project Title: Genetic Analysis of *Phragmites australis*, Wupatki National Monument

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CPCESU Partner Institution: Northern Arizona University

Principal Investigator: Dr. Gery Allan, Associate Prof., Director EnGGen Facility, 500 S. Beaver ST., Bldg 21, Box 5640
Phone: (928) 523-8934
e-mail: gery.allan@nau.edu

NPS Key Official: Paul Whitefield, Natural Resource Specialist
Flagstaff Area National Monuments
6400 N Highway 89, Flagstaff AZ 86004
Phone: (928) 526-1157 ext. 235
e-mail: paul_whitefield@nps.gov

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Introduction

Phragmites australis (common reed) is a perrenial grass that is frequently found in marsh communities associated with lakes, rivers and ponds distributed throughout North America. Although *P. australis* is believed to be native to North America, an exotic strain of this species was first identified along the Atlantic coast and is believed to be of European origin (Saltonstall 2002, Chambers et al. 1999). As part of an ongoing restoration effort at Wupatki National Monument (WNM), the Environmental Genetics & Genomics (EnGGen) laboratory, under the direction of Dr. Gery Allan, agreed to investigate the genetic origins of *P. australis* found at Peshlakai Spring within WNM. The overall goal of this study was to determine if *P. australis* at Peshlakai was genetically contaminated with a non-native strain (i.e, exotic genotype), which is now found throughout the mainland United States. Using molecular markers (microsatellites) and DNA sequences that have been shown to be useful for distinguishing between native and non-native *Phragmites* genotypes (Saltonstall 2002, 2003), we examined plants collected from Peshlakai Spring in order to determine if they are of native or non-native origin. Native and non-native strains of *P. australis* were originally

investigated by Saltonstall (2002, 2003) who showed that the two strains could be distinguished based on molecular markers derived from nuclear microsatellites and chloroplast DNA sequences.

Methods

Sampling - Leaf samples from fifty individual stems were collected from Peshlakai Springs in WNM. Given that *Phragmites* reproduces both sexually and asexually (via underground runners), stems were sampled from as many "clumps" as possible. However, whether individual stems represented individual ramets (individual plants) or genets (clonal group of individuals) was not known. We also received 10 additional stems from a small population in Arches National Park, Utah (via Charles Schelz). We obtained five known known native and five non-native samples from Cornell University to serve as controls. Leaf tissue from all samples was dried using silica gel and stored in airtight containers.

DNA extractions – DNA was extracted from all samples using a Qiagen DNeasy Extraction Kit. DNA from each extraction was stored in 1.5 ml microfuge tubes and quantified using nanodrop fluorometry. Isolated DNAs were then transferred to a 96 well plate for further analysis. **Table 1** shows sample identities for all collected and received samples.

Microsatellite Genotyping – Using microsatellite loci developed by Saltonstall (2003), we chose three loci for genetic fingerprinting of all samples. These loci were identified as PaGT9, PaGT11 and PaGT14, and consist of dinucleotide repeats (2 bp "CA" repeats) repeated multiple times at each locus (see Saltonstall 2003, Table 1 pg 1692). **Table 2** shows the primer sequences, associated repeat units and expected number of alleles for each locus. The following final concentrations were used for each PCR reaction (10uL) to amplify each of the three loci: 1X PCR buffer; 1.5mM MgCl2; 0.5mM DNTPs; 0.12uM of each primer and; 0.5U of *Taq* polymerase. Reactions were amplified using a PCR program with denaturation for 4 min @ 95C, followed by 35 iterations of 95C for 30 sec, 55C for 30 sec and 72C for 2 min. A final extension step was implemented for 7 min @ 72C. A LIZ size standard was included with each reaction and reactions were performed in 96 well plates on a BioRad iCycler.

DNA Sequencing – Two noncoding regions within the chloroplast genome (cpDNA) were targeted for sequencing as per the strategy used by Saltsonstall (2002). Each region was PCR-amplified by using the primer pairs trnT(UGU) ''a''–trnL(UAA)5 ''b'' (26) and rbcL–psaI (27) with annealing temperatures of 56°C and 54°C, respectively. Double-stranded PCR amplifications were sequenced directly in both directions on an Applied Biosystems 3730 sequencer using the amplification primers and two internal primers in the rbcL-psaI region (rpL23F 5 - AGGTAGTAGCTGTGAATAGC and rpL23R 5 -AGTCGAT-GGCTATTCACAGC).

Data Analysis

MSAT Genotyping — Scoring of microsatellite alleles was performed using Genemapper software, which sizes each allele according to a specified LIZ size standard. Raw data was then compiled and analyzed using GenAlEx genotyping software. A distance-based cluster diagram was generated using the program MEGA v5.0.

DNA Sequencing — DNA sequence alignment and editing was performed using the LaserGene software package with the SeqMan and MegAlign sub-programs. DNA sequence analyis was conducted using MEGA v5.0.

Results

MSAT Genotyping — We compared our genotyping results with that of Saltonstall (2003), as well as the control samples (native and non-native) received from Cornell University. Our analyses revealed a total of 15 alleles in the Wupatki and Arches samples combined. Locus PaGT9 had the highest number of alleles (6), followed by PaGT14 (5) and PaGT11 (4). Allele sizes ranged from 141 base pairs (bp) to 207bp, which is consistent with previous analyses (Saltonstall 2003). Alleles 188bp, 199bp and 207bp were unique to locus PaGT9. One allele (147 bp) was unique to PaGT11 and two alleles (177bp and 189bp) were unique to PaGT14. Of these, alleles 147bp, 177bp, 188bp, 189bp and 207bp were only found in association with native *Phragmites* genotypes. Allele 199bp was unique to one sample of non-native *Phragmites*. In addition to the alleles described above other alleles were also identified that were shared among both native and non-native samples. The sharing of alleles between the two strains is not unexpected since the native and non-native taxa are recently derived and share a high proportion of their genomes. Hence, in order to distinguish native from non-native genotypes, we only evaluated those alleles that were diagnostic for either known native, or known non-native samples (**Table 3**).

Percent polymorphic loci ranged from 0.0% in the Arches samples to 33.3% (Wupatki), with a high of 100% in both the known native and non-native samples. Mean observed heterozygosity ranged from 0.0 (Arches samples) to 1.67 (Wupatki samples) to 0.481 (known native) and 0.667 in known non-native samples. The low observed heterozygosity for the Arches samples could be due to the collection consisting of individual ramets of a single genet, which itself is homozygous. The lack of heterozygous genotypes is also consistent with the lack of polymorphic loci for these samples.

A genetic cluster diagram (**Fig. 1**) shows that the Wupatki and Arches samples (represented by salmon- and green-colored triangles, respectively) are each other's closest relatives (i.e., they form a single clade). These samples, in turn, are most closely related to known native *Phragmites* control samples (sky blue rectangle). The most distantly related samples belong to separate lineages

comprising all of the known non-native *Phragmites* samples (light blue rectangle). Hence, for the purposes of this report, it is important to note that the Wupatki and Arches samples form distinct lineages that, together, are most closely related to known native *Phragmites*. This information is corroborated by separate analyses of genetic distance (not shown), which show that the smallest genetic distance is between the Wupatki and Arches samples, while the greatest genetic distance is between these samples and known non-native *Phragmites*.

DNA Sequencing — The two chloroplast DNA regions, RbcL and TrnT, yielded 480 and 804 basepairs, respectively. Sequence divergence for both regions was low for both the within control groups and the Wupatki and Arches samples. However, as per Saltonstall's analysis (2003) some differences were observed either in the form of nucleotide substitutions or "gaps" (insertions or deletions) in the data set. **Table 4** shows two variable positions for RbcL and three variable positions for TrnT. As with the MSAT data, differences in the DNA sequence data set were diagnostic for some, but not all sample categories (native vs. non-native). For example, Table 4 shows overlap for non-native and native samples at positions 31 and 278 for the RbcL region and each of the three positions for the TrnT region. Nevertheless, three positions (highlighted in gray) showed the same result for a set of known native and the Wupatki and Arches samples (position 31, RbcL; positions 612, 672; TrnT). Two additional positions (highlighted in light blue) also showed a shared similarity among known native and the Wupatki and Arches samples. It should also be noted that unlike the MSAT data set, no sequence differences (in either region) were seen among the Wupatki and Arches samples. This is not surprising given the highly conserved nature of the chloroplast genome, which has a much lower mutation rate than nuclear MSAT markers.

Discusssion

Our analyses strongly suggest, but do not conclusively prove, that the Wupatki and Arches samples are free from contamination with non-native *Phragmites* germplasm. The MSAT data set clearly shows overlap in allelic composition between known native/non-native samples and the Wupatki and Arches samples. Nevertheless, the Wupatki and Arches samples exhibit diagnostic differences from known non-native *Phragmites* (Table 3) for two of the three loci. This, together with the stated "native" morphology of the Wupatki and Arches samples (Paul Whitefield pers. comm) suggests that these samples are likely of native origin.

The DNA sequence data from two independent chloroplast regions shows similar results to those of the MSAT data set. Despite the low sequence variability of these regions, differences between native and non-native samples exist, along with some overlap (shared similarities) among native and non-native samples. Moreover, the differences that do exist vary to some degree in the number of known native samples that show similarities to the Wupatki and Arches samples. For example, in a comparison among positions 31, 612 & 672

and positions 278 & 110 (Table 4) it is clear that different subsets of known native and Wupatki-Arches samples have shared similarities (i.e, the shared similarities are not consistent across all known native and the Wupatki-Arches samples). This, however, is not altogether unusual given the large number of shared similarities (i.e., no differences) among the two regions across all samples (only 2 nucleotide subsititions were observed in the RbcL region and only 3 in the TrnT region out of a total of 1,284 nucleotide positions). Nevertheless, given the consistent results between the DNA sequence data and the MSAT data set it appears that the Wupatki and Arches samples are native in origin. It should also be noted that among the known native samples four samples (Berg_1, Berg_2 & Diep_1, Diep_2) consistently showed 100% similarity to the Wupatki and Arches samples for both the DNA and MSAT data sets.

Overall, our analyses show that the Wupatki and Arches samples are more genetically similar to known native *Phragmites* than to non-native *Phragmites*. These results suggest (but do not prove) that the genetic integrity of *P. australis* in WNM and ANP has not been compromised and is therefore a useful resource for restoration within our southwestern national parks.

Finally, it should be noted that based on the MSAT results, the Wupatki and Arches populations exhibit some allelic differences (e.g., allele 143 is present in Wupatki samples, but was not observed in the Arches samples), as well as genotypic differences (Wupatki samples show more heterozygous genotypes, whereas the Arches samples are homozygous at all loci examined; Table 4). These differences in allelic and genotypic composition suggests that the two populations could be interbred to increase genetic diversity in restoration projects. It should also be understood, however, that although we sampled intensively at WNM, the extent of the population size (and therefore sampling intensity) at ANP is not known. Hence, in order to better understand the extent to which the two populations are different in their genetic composition, more thorough sampling of *Phragmites* at ANP is recommended.

References

Chambers, R.M., L.A. Meyerson and K. Saltonstall (1999). Expansion of *Phragmites australis* into tidal wetlands of North America. *Aquatic Botany* **64**:261-273

Saltonstall K. (2002) Cryptic invasion by a non-native genotype of *Phragmites australis* into North America. *Proceedings of the National Academy of Sciences, USA* **99**:2445-2449.

Saltonstall, K. (2003). Microsatellite variation within and among North American lineages of Phragmites australis. *Molecular Ecology*, **12**: 1689-1702.

Figures & Tables

Fig. 1. Genetic cluster analysis of known native and non-native samples and the Wupatki and Arches samples based on three MSAT loci.

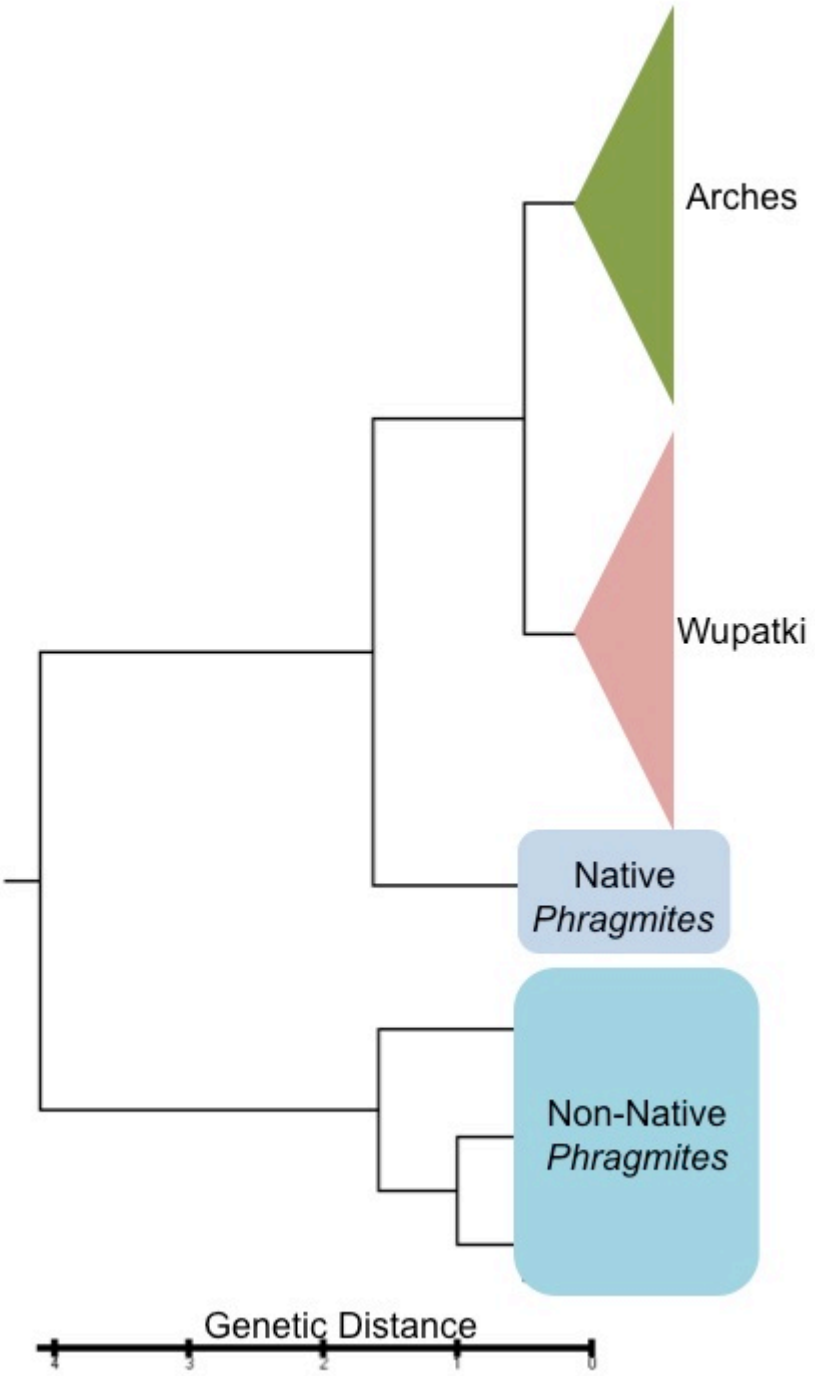

Table 1. Sample Identities and Origin

Sample Name		Origin
Cornell Control Samples	Ant_1	Known Native Genotype
	Ant_2	Known Native Genotype
	Berg_1	Known Native Genotype
	Berg_2	Known Native Genotype
	Diep_1	Known Native Genotype
	Diep_2	Known Native Genotype
	PW_1	Known Native Genotype
	PW_2	Known Native Genotype
	LL_1	Known Non-Native Genotype
	LL_2	Known Non-Native Genotype
	M59_1	Known Non-Native Genotype
	M59_2	Known Non-Native Genotype
	MZ1_a	Known Non-Native Genotype
	MZ1_b	Known Non-Native Genotype
	MZ2_a	Known Non-Native Genotype
	Nov_1	Known Non-Native Genotype
	Nov_2	Known Non-Native Genotype
	Roch_1	Known Non-Native Genotype
	Roch_2	Known Non-Native Genotype
Wupatki Samples	Pes1_1	Native phenotype
	Pes1_2	Native phenotype
	Pes1_3	Native phenotype
	Pes1_3_2	Native phenotype
	Pes1_4	Native phenotype
	Pes1_5	Native phenotype
	Pes1_5	Native phenotype
	Pes2_1	Native phenotype
	Pes2_2	Native phenotype
	Pes2_3	Native phenotype
	Pes2_4	Native phenotype
	Pes2_5	Native phenotype
	Pes3_1	Native phenotype
	Pes3_2	Native phenotype
	Pes3_3	Native phenotype
	Pes3_4	Native phenotype
	Pes3_4	Native phenotype
	Pes3_5	Native phenotype
	Pes4_1	Native phenotype
	Pes4_2	Native phenotype
	Pes4_2_2	Native phenotype
	Pes4_3	Native phenotype
	Pes4_4	Native phenotype
	Pes4_5	Native phenotype

	Pes5_1	Native phenotype
	Pes5_2	Native phenotype
	Pes5_3	Native phenotype
	Pes5_4	Native phenotype
	Pes5_4_2	Native phenotype
	Pes5_5	Native phenotype
	Pes6_1	Native phenotype
	Pes6_2	Native phenotype
	Pes6_3	Native phenotype
	Pes6_4	Native phenotype
	Pes6_4_2	Native phenotype
	Pes6_5	Native phenotype
	Pes7_1	Native phenotype
	Pes7_2	Native phenotype
	Pes7_3	Native phenotype
	Pes7_4	Native phenotype
	Pes7_5	Native phenotype
	Pes7_5_2	Native phenotype
	Pes8_1	Native phenotype
	Pes8_2	Native phenotype
	Pes8_3	Native phenotype
	Pes8_4	Native phenotype
	Pes8_5	Native phenotype
	Pes9_1	Native phenotype
	Pes9_2	Native phenotype
	Pes9_3	Native phenotype
	Pes9_4	Native phenotype
	Pes9_5	Native phenotype
Arches Samples	**Utah_1**	Native phenotype
	Utah_10	Native phenotype
	Utah_10_2	Native phenotype
	Utah_2	Native phenotype
	Utah_2	Native phenotype
	Utah_3	Native phenotype
	Utah_4	Native phenotype
	Utah_4_2	Native phenotype
	Utah_5	Native phenotype
	Utah_6	Native phenotype
	Utah_7	Native phenotype
	Utah_8	Native phenotype
	Utah_9	Native phenotype
	Utah_9	Native phenotype

Table 2. Primer sequences, repeat units, alleles and allele sizes.

MSAT Primer	Sequence (5'-3')	No. Repeat units	Max no.	Allele size range (bp)
PaGT9_F	CCATGTGTTAATGTTGTCC	(CA)10	3	188-224
PaGT9_R	ATTGAATCCACACGTTTCCG			
PaGT11_F	CAACTCCGTGAATGACATGC	(CA)8	3	142-151
PaGT11_R	CAGTTTGTGCACTAATGGAC			
PaGT14_F	GTTGCAGCAAGTATTTGG	(CA)7	4	169-198
PaGT14_R	CAAGCATTCTAGTAGTAGC			

Table 3. MSAT loci and alleles for each locus (allele sizes are in base pairs). Although some overlap in allele types occurs among known native and non-native samples (e.g, in locus PaGT11), note that for locus PaGT9 the 203 bp allele in the Peshlakai and Arches samples is only found in the known native samples. Similarly, the 173 allele for locus PaGT14 is only found in the known native samples. In a few cases a third allele was found in the control samples for locus PaGT11 (e.g., alleles 147, 149), suggesting that these samples are likely triploids. This finding, however, has no relevance for the Wupatki and Arches samples as no triploids were identified.

	Sample Identity	PaGT9		PaGT11			PaGT14		
K. Saltonstall Dominant Phenotypes	Introduced	198	198	142	147		189	189	
		198	202	142	147	149	183	189	
							187	189	
	Native	210	210	145	145		181	181	
		210	212						
	Gulf Coast	192	196	142	147		177	189	
				142	145	147	177	185	189
	Europe	198	204	142	142		181	181	
		198	206	142	147		189	189	
							187	189	
Cornell Control Samples	Ant_1	188	192	141	145		177	189	
	Ant_2	188	192	141	145		177	189	
	Berg_1	203	203	143	143		173	173	
	Berg_2	203	203	143	143		173	173	
	Diep_1	203	207	143	143		173	173	
	Diep_2	203	207	143	143		173	173	
	MZ2_a	192	196	141	147		180	182	
	PW_1	192	192	141	145		182	182	
	PW_2	192	192	141	145		182	182	
	LL_1	196	196	141	145	147	182	182	
	LL_2	196	196	141	145	147	182	182	
	M59_1	192	192	141	145	147	180	182	
	M59_2	192	192	141	145	147	180	182	
	MZ1_a	196	199	141	145		180	182	
	MZ1_b	196	199	141	145		180	182	
	Nov_1	192	196	141	145	147	180	182	
	Nov_2	192	196	141	145	147	180	182	
	Roch_1	192	192	141	145	147	182	182	
	Roch_2	192	192	141	145	147	182	182	

Known Native Genotype

Known Non-Native Genotype

(Table 3 cont'd)

	Sample Identity	PaGT9		PaGT11			PaGT14		
Wupatki Samples	Pes1_1	203	203	141	143		173	173	
	Pes1_2	203	203	141	143		173	173	
	Pes1_3	203	203	141	143		173	173	
	Pes1_3_2	203	203	141	143		173	173	
	Pes1_4	203	203	141	143		173	173	
	Pes1_5	203	203	141	143		173	173	
	Pes1_5	203	203	141	143		173	173	
	Pes2_1	203	203	141	143		173	173	
	Pes2_2	203	203	141	143		173	173	
	Pes2_3	203	203	141	143		173	173	
	Pes2_4	203	203	141	143		173	173	
	Pes2_5	203	203	141	143		173	173	
	Pes3_1	203	203	141	143		173	173	
	Pes3_2	203	203	141	143		173	173	
	Pes3_3	203	203	141	143		173	173	
	Pes3_4	203	203	141	143		173	173	
	Pes3_4	203	203	141	143		173	173	
	Pes3_5	203	203	141	143		173	173	
	Pes4_1	203	203	141	143		173	173	
	Pes4_2	203	203	141	143		173	173	
	Pes4_2_2	203	203	141	143		173	173	
	Pes4_3	203	203	141	143		173	173	
	Pes4_4	203	203	141	143		173	173	
	Pes4_5	203	203	141	143		173	173	
	Pes5_1	203	203	141	143		173	173	
	Pes5_2	203	203	141	143		173	173	
	Pes5_3	203	203	141	143		173	173	
	Pes5_4	203	203	141	143		173	173	
	Pes5_4_2	203	203	141	143		173	173	
	Pes5_5	203	203	141	143		173	173	
	Pes6_1	203	203	141	143		173	173	
	Pes6_2	203	203	141	143		173	173	
	Pes6_3	203	203	141	143		173	173	
	Pes6_4	203	203	141	143		173	173	
	Pes6_4_2	203	203	141	143		173	173	
	Pes6_5	203	203	141	143		173	173	
	Pes7_1	203	203	141	143		173	173	
	Pes7_2	203	203	141	143		173	173	
	Pes7_3	203	203	141	143		173	173	
	Pes7_4	203	203	141	143		173	173	
	Pes7_5	203	203	141	143		173	173	
	Pes7_5_2	203	203	141	143		173	173	
	Pes8_1	203	203	141	143		173	173	
	Pes8_2	203	203	141	143		173	173	
	Pes8_3	203	203	141	143		173	173	
	Pes8_4	203	203	141	143		173	173	
	Pes8_5	203	203	141	143		173	173	
	Pes9_1	203	203	141	143		173	173	
	Pes9_2	203	203	141	143		173	173	
	Pes9_3	203	203	141	143		173	173	
	Pes9_4	203	203	141	143		173	173	
	Pes9_5	203	203	141	143		173	173	

	Sample Identity	PaGT9		PaGT11			PaGT14		
Arches Samples	Arches_1	203	203	141	141		173	173	
	Arches_10	203	203	141	141		173	173	
	Arches_10_2	203	203	141	141		173	173	
	Arches_2	203	203	141	141		173	173	
	Arches_2	203	203	141	141		173	173	
	Arches_3	203	203	141	141		173	173	
	Arches_4	203	203	141	141		173	173	
	Arches_4_2	203	203	141	141		173	173	
	Arches_5	203	203	141	141		173	173	
	Arches_6	203	203	141	141		173	173	
	Arches_7	203	203	141	141		173	173	
	Arches_8	203	203	141	141		173	173	
	Arches_9	203	203	141	141		173	173	
	Arches_9	203	203	141	141		173	173	

Table 4. DNA sequencing results for two chloroplast DNA regions, RbcL and TrnT. Postion numbers refer to nucleotide positions in the aligned data set. Nucleotide differences are color-coded, while gaps in the data set are simply stated as a "gap." Gaps typically correspond to insertions or deletions of nucleotides as a result of mutation in non-coding DNA regions. Note that only a subset of Wupatki and Arches samples are shown because sequences for these samples were identical within each of the two sampled populations.

	RbcL		trnT			
Sample	**Pos. 31**	**Pos. 278**	**Pos. 110**	**Pos. 612**	**Pos. 672**	**Origin**
LL_1	C	gap	gap	gap	gap	Non-native
LL_2	C	gap	gap	gap	gap	Non-native
M59_1	C	gap	gap	gap	gap	Non-native
M59_2	C	gap	gap	gap	gap	Non-native
MZ_1	C	gap	gap	gap	gap	Non-native
MZ1_b	C	gap	gap	gap	gap	Non-native
Nov_1	C	gap	gap	gap	gap	Non-native
Nov_2	C	gap	gap	gap	gap	Non-native
Roch_1	C	gap	gap	gap	gap	Non-native
Roch_2	C	gap	gap	gap	gap	Non-native
MZ2_a	C	gap	gap	gap	gap	Non-native
PW_1	C	gap	gap	gap	gap	Native
PW_2	C	gap	gap	gap	gap	Native
Ant_1	A	gap	gap	A	G	Native
Ant_2	A	gap	gap	A	G	Native
Berg_1	A	T	TTAGAAAA	A	G	Native
Berg_2	A	T	TTAGAAAA	A	G	Native
Diep_1	A	T	TTAGAAAA	A	G	Native
Diep_2	A	T	TTAGAAAA	A	G	Native
Pes1-1	A	T	TTAGAAAA	A	G	Native
Pes1-2	A	T	TTAGAAAA	A	G	Native
Pes1-4	A	T	TTAGAAAA	A	G	Native
Pes1-5	A	T	TTAGAAAA	A	G	Native
Pes2-1	A	T	TTAGAAAA	A	G	Native
Arches-2	A	T	TTAGAAAA	A	G	Native
Arches-5	A	T	TTAGAAAA	A	G	Native
Arches-6	A	T	TTAGAAAA	A	G	Native
Arches-8	A	T	TTAGAAAA	A	G	Native
Arches-9	A	T	TTAGAAAA	A	G	Native
Arches-10	A	T	TTAGAAAA	A	G	Native