Isolation and characterization of novel polymorphic microsatellite markers for the white stork, *Ciconia ciconia*: applications in individual–based and population genetics

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Abstract

Isolation and characterization of novel polymorphic microsatellite markers for the white stork, Ciconia ciconia: *applications in individual–based and population genetics*.— The white stork, *Ciconia ciconia*, is a model species for studies of bird migration and behavior, but previously published genetic markers are not informative enough to perform individual–based genetic studies. Following discovery using next generation sequencing, 11 polymorphic markers were selected and tested in samples from two study sites. The number of alleles per locus ranged from 2–10 with an average of 5.3. The mean observed and expected heterozygosities were 0.519 and 0.565 respectively. P_{ID} was adequately sensitive for population– and individual–based genetic studies. There was no significant evidence of allelic drop–out, null alleles, or other errors; one sample site deviated from Hardy–Weinberg equilibrium for two loci, but no loci deviated in both samples, suggesting utility of these markers. These markers can be used to answer a range of ecological questions including those related to genetic diversity, degree of natal philopatry, and genetic mating strategies.

Key words: Genetic markers, Short tandem repeats, Relatedness, Probability of identity, Polymorphism, Genetic diversity

Resumen

Aislamiento y caracterización de nuevos marcadores de microsatélites polimórficos para la cigüeña blanca, Ciconia ciconia: *aplicaciones de la genética basada en individuos y de poblaciones*.— A pesar de que la cigüeña blanca, *Ciconia ciconia,* es una especie modelo en estudios de migración y comportamiento, los marcadores moleculares publicados hasta ahora no son lo suficientemente polimórficos para poder realizar estudios genéticos basados en el individuo. Utilizando la secuenciación de nueva generación hemos seleccionado 11 marcadores polimórficos y los hemos utilizado en cigüeñas de dos localidades de estudio. El número medio de alelos por locus fue de 5,3 con un mínimo de dos y un máximo de diez. La heterocigosidad media observada y esperada fue de 0,519 y 0,565, respectivamente. La P_{ID} (probabilidad de identidad) resultó ser suficientemente sensible para los estudios sobre genética basada en individuos y genética de poblaciones. No hemos encontrado evidencias de pérdida alélica, alelos nulos ni ningún otro error, y ningún locus estaba en desequilibrio de Hardy–Weinberg en ambas localidades a pesar de que dos loci sí que lo estuvieran en una única localidad. Estos marcadores son útiles para dar respuesta a una serie de preguntas relacionadas con la diversidad genética, el grado de filopatria y las estrategias genéticas de reproducción.

Palabras clave: Marcadores moleculares, Repeticiones cortas en tándem, Parentesco, Probabilidad de identidad, Polimorfismo, Diversidad genética

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Introduction

The white stork, *Ciconia ciconia*, is a model species for studies of bird migration and behavior because of its long life span, suspected monogamy and philopatry, proximity to human settlements, ubiquity, ease of identification, and magnificent migratory journeys. Much is known about the species through observation and biotelemetry tracking, although these approaches might be biased (ring–resightings) or suitable for just a few tens of individuals because of high monetary investments and associated costs (*e.g.,* tracking: data download, GPS trackers). While one population genetics study has been performed (Shephard et al., 2013), the mean number of alleles for the 18 populations considered was very low: 3.01 ± 0.75 (mean ± SD); furthermore, little is known about genetic mating strategies, nest fidelity, and levels of natal philopatry in white storks although these behaviors are relatively easy to study using a powerful set of molecular markers. Therefore, coordination between international research groups at the time of ringing (an annual occurrence at thousands of nests throughout Europe and the Middle East) in order to collect genetic samples (feather collection) and use of a highly polymorphic microsatellite panel can provide vast data useful for a variety of ecological and behavioral studies.

A preliminary test of microsatellite markers published by Shephard et al. (2009), using genetic material from wild individuals, showed even lower polymorphism levels than originally reported. From the initial (published) panel of 13 microsatellites, only seven remained after removing markers where null alleles (n = 2), linkage disequilibrium (n = 1), and amplification issues (n = 3) were found. This reduced panel of largely dimorphic markers is insufficient to elucidate individual behavioral strategies and population dynamics in this model species. The development of new markers, pivotal to future genetic–based studies, is therefore essential. Here, we present 11 polymorphic markers —selected and tested in samples from two wild study sites— that can be used to answer a range of ecological questions on white storks.

Material and methods

We developed microsatellites using a next generation sequencing approach performed by *EcoGene NZ: DNA–based diagnostics* (Auckland, New Zealand) in 2013 from DNA extracted from blood samples of two white storks, one from a wild population in northern Israel and one from a wild population in northeast Germany. We discovered over 100 potentially polymorphic microsatellite loci with a throughput of 35M bases, an average read length of 441.6 base pairs, and a total of 170,969 reads (64,583 and 106,139 reads per run, respectively); primers were designed by *EcoGene NZ* using *msatcommander* (Faircloth, 2008).

Sixty–four microsatellite loci were chosen based on size (optimal length was considered as 100–350 base pairs) and number of bases repeated (loci with tetra–base repeat motifs were preferred). These 64 markers were tested for amplification and polymorphism with PCR using the M13 method for fluorescent labeling (Schuelke, 2000) in a subset of 94 stork samples (see below for sampling information). PCR was performed in 20 µL volumes with 2.7 µL DNA (1:20 dilution of an NaOH extraction; Zhang et al., 1994), 10 µL Taq Plus Master Mix 2x (Lamda Biotech; contains 1.5 mM $MgCl_2$), 0.5 µL (final concentration: 0.25 µM) fluorescent–labeled M13 (either 6FAM or TAMRA), 0.5 µL (0.25 µM) reverse primer, 0.342 µL (0.0175 µM) forward primer, and 5.96 µL double distilled water. PCR conditions were as follows: an initial step at 94°C for five minutes followed by a 'touchdown' cycling program of 16 cycles with 92°C for 30 seconds; annealing for 30 seconds, starting at 60°C and decreasing by 1°C for each of the 16 cycles to 45°C; and 72°C for 30 seconds, followed by 30 cycles continued at an annealing temperature of 45°C, all followed by a final step at 72°C for 10 min. We also applied a similar 'touchdown' cycling from 55ºC to 45ºC with the last 30 cycles at an annealing temperature of 45ºC and/or increased $MgCl_2$ concentrations to 2.5 mM for those loci that did not amplify (see table 1).

Genotyping was performed using an ABI PRISM™ 3730 xl DNA Analyzer by the Hebrew University Center for Genomic Technologies (Jerusalem, Israel). Allele calling and binning were obtained using *GeneMap per 4.0* software (Applied Biosystems, Foster City, CA, USA). Of the 64 loci initially tested, 11 loci were selected based on consistency of amplification and number of alleles per locus (Cc10, Cc15, Cc18, Cc37, Cc42, Cc44, Cc50, Cc58, Cc61, Cc69, and Cc72), and PCR conditions for these loci were then further optimized (see table 1).

Following marker selection, we genotyped 213 individuals using the NaOH extraction method mentioned above. Feathers (five) were collected from juveniles from two sample sites prior to fledging; only one individual per nest was included in this analysis. Samples were collected in 2012 from northeast Germany (n = 152; center point of sampling: 52.7383º N, 11.6681º E) and in 2015 from eastern Greece (n = 61; center point of sampling: 41.0520º N, 25.1223º E). Following PCR amplification, genotyping, and scoring (as described above), and tests of Hardy–Weinberg equilibrium (*Cervus 3.0.3*; Kalinowski et al., 2007) were performed as were tests of genotyping error (*GIMLET*; Valière, 2002), null alleles (*FreeNA*; Chapuis & Estoup, 2007), allelic drop–out (*GIMLET*), inbreeding (F_{IS}; *Genetix 4.05.2;* Belkhir et al., 2004), linkage disequilibrium (*GENEPOP* with Bonferroni correction for significance; Raymond & Rousset, 1995; Rousset, 2008), and genetic structure (F_{ST}; *Genetix 4.05.2*). Rates of expected and observed heterozygosities, the mean polymorphic information content (PIC) and P_{ID} and $P_{ID–Sib}$ (probability of identity, the likelihood that two unrelated or sibling–related individuals, respectively, will have the same genotype profile by chance) were also calculated (*Cervus 3.0.3*).

Table 1. Characterization of 11 species–specific microsatellite loci with conditions for PCR optimization based on 213 individuals from Germany (n = 152) and Greece (n = 61). Primer sequences (F. Forward, R. Reverse; *M13 Sequence: 5'–TGTAAAACGACGGCCAGT–3'); RM. Repeat motif; GB. GeneBank accession number; TD. 'Touchdown' annealing temperatures (ºC); T[$MgCl_2$]. Final $MgCl_2$ concentration in PCR reaction (mM); SR. Size range (bp); Na. Number of alleles; H_{Obs}. Observed heterozygosity; H_{Exp}. Expected heterozygosite; PIC. Polymorphic information content (PIC).

*Tabla 1. Caracterización de 11 loci de microsatélites específicos de una especie con condiciones de optimización de la PCR basada en 213 individuos de Alemania (n = 152) y Grecia (n = 61). Secuencias del cebador (F. Adelante, R. Atrás; *Secuencia de M13: 5'–TGTAAAACGACGGCCAGT–3'); RM. Secuencia repetida; GB. Número de acceso a GeneBank; TD. temperaturas de hibridación de la PCR con rampa decreciente de temperaturas; T[$MgCl_2$]. Concentración final de $MgCl_2$ en la PCR (mM); SR. Intervalo de longitud (pares de bases); Na. Número de alelos; H_{Obs}. Heterocigosidad observada; H_{Exp}. Heterocigosidad esperada; PIC. Índice de contenido polimórfico.*

Locus	primer sequences (5'–3')								
	RM	GB	TD	T[$MgCl_2$]	SR	Na	H_{Obs}	H_{Exp}	PIC
Cc10	F–M13–TGTGACAGATGCAAAGCTCC				R–GTGTTTACTAGTTGGCTGTTCC				
	(AGAT)9	KT232056	60–45	1.5	95–147	7	0.784	0.804	0.774
Cc15	F–M13–ATAGCAACGATGTTCCACCC				R–AAACCCAGCTTTGTTCTGCC				
	(AAAC)8	KT232057	60–45	1.5	155–159	2	0.118	0.136	0.127
Cc18	F–M13–AGGGTGGTTATGTGCTCAGG				R–ACTCTGACTGGGATGTGCTC				
	(ACAT)6	KT232058	60–45	1.5	237–287	2	0.468	0.471	0.359
Cc37	F–M13–CCTGCCTGACAAGAGAATGC				R–GCAAGTGTATCAGTCCAAATGG				
	(AC)8	KT232059	60–45	1.5	244–252	5	0.512	0.57	0.485
Cc42	F–M13–GCAGGAAAGGAGGAAAGGTG				R–GCATCACAGTATGCAAACGC				
	(AGAT)8	KT232060	60–45	2.5	286–346	7	0.49	0.468	0.437
Cc44	F–M13–TGCATCCTTTGTCTTGCCAG				R–CTGCCCTCCTGATATGTCCC				
	(ACAG)6	KT232061	60–45	1.5	331–339	3	0.525	0.524	0.429
Cc50	F–M13–CTAATCTGTCCTGCCCTCCC				R–CACAGAGCCAGCAAGACAAG				
	(AC)10	KT232062	60–45	2.5	209–215	5	0.335	0.448	0.42
Cc58	F–M13–ACGAGGGTTGCTTAAGGAGG				R–AAATCTGTGCGCCAACTCAC				
	(AC)16	KT232063	60–45	2.5	245–265	10	0.662	0.768	0.729
Cc61	F–M13–GCTGCCTGACCAAGAGAAAC				R–TCCTGCTTGTTTCCTTCCTC				
	(AC)9	KT232064	55–45	2.5	265–271	3	0.545	0.572	0.476
Cc69	F–M13–ACAATGCCTGGACCACAATG				R–CTCATTCTTGGCACGAACCC				
	(AT)12	KT232065	55–45	2.5	312–322	8	0.638	0.827	0.802
Cc72	F–M13–CATTGAAGATACTGGGCAGCC				R–GATCCCTTCATCACCAGCAG				
	(AT)8	KT232066	60–45	1.5	216–228	6	0.634	0.621	0.569
Mean						5.3	0.519	0.565	0.510

Results

Overall polymorphism for the two samples together ranged from two to ten alleles per locus, with a mean of 5.3 alleles and a median of 5 (see table 1 for overall values; see table 2 for data regarding the two populations). Observed and expected heterozygosities ranged from 0.118–0.784 and 0.136–0.827, respectively, with overall mean observed and expected heterozygosities of 0.519 and 0.565. The mean PIC was 0.510 and the P_{ID} and $P_{ID–Sib}$ were 0.00000005 and 0.0007678, respectively. Two markers, Cc15 and Cc18, had low polymorphism but had a positive influence on P_{ID} (see Discussion). Removing

Table 2. Summary of locus diversity (L) and panel specificity per study site: SR. Sample region (Ge. Germany, Gr. Greece); Na. Number of allelles; H_{Obs}. Observed heterozygosity; H_{Exp}. Expected heterozygosity; PIC. Polymorphic information content; HWE. Deviations from Hardy–Weinberg equilibrium (NS. Not significant, ND. Not performed; * 0.01 < *p* < 0.05; ** 0.001 < *p* < 0.01; *** *p* < 0.001); Ni. Number of individuals; MT. Mean proportion typed.

*Tabla 2. Resumen de la diversidad de locus (L) y especificidad del panel por lugar de estudio: SR. Región de la muestra (Ge. Alemania, Gr. Grecia); Na. Número de alelos; H_{Obs}. Heterocigosidad observada; H_{Exp}. Heterocigosidad esperada; PIC. Índice de contenido polimórfico; HWE. Desviaciones del equilibrio de Hardy–Weinberg (NS. No significativo, ND. No realizado; * 0,01 < p < 0,05; ** 0,001 < p < 0,01; *** p < 0,001); Ni. Número de individuos; MT. Proporción media de individuos genotipados.*

L	SR	Na	H_{Obs}	H_{Exp}	PIC	HWE
Cc10	All	7	0.784	0.804	0.774	NS
	Ge	7	0.816	0.810	0.780	NS
	Gr	7	0.705	0.785	0.745	NS
Cc15	All	2	0.118	0.136	0.127	ND
	Ge	2	0.079	0.101	0.095	ND
	Gr	2	0.217	0.221	0.195	ND
Cc18	All	2	0.468	0.471	0.359	NS
	Ge	2	0.483	0.457	0.352	NS
	Gr	2	0.429	0.499	0.372	NS
Cc37	All	5	0.512	0.570	0.485	NS
	Ge	5	0.500	0.585	0.507	NS
	Gr	4	0.542	0.526	0.410	NS
Cc42	All	7	0.490	0.468	0.437	NS
	Ge	7	0.470	0.458	0.423	NS
	Gr	7	0.544	0.497	0.466	NS
Cc44	All	3	0.525	0.524	0.429	NS
	Ge	3	0.549	0.515	0.432	NS
	Gr	3	0.466	0.521	0.400	NS
Cc50	All	5	0.335	0.448	0.420	**
	Ge	5	0.333	0.449	0.420	*
	Gr	3	0.339	0.426	0.374	ND
Cc58	All	10	0.662	0.768	0.729	***
	Ge	10	0.685	0.779	0.743	***
	Gr	8	0.603	0.723	0.666	NS
Cc61	All	3	0.545	0.572	0.476	NS
	Ge	3	0.577	0.575	0.479	NS
	Gr	3	0.467	0.561	0.464	NS
Cc69	All	8	0.638	0.827	0.802	***
	Ge	6	0.698	0.757	0.713	NS
	Gr	6	0.479	0.676	0.610	NS
Cc72	All	6	0.634	0.621	0.569	NS
	Ge	6	0.699	0.651	0.594	NS
	Gr	6	0.464	0.513	0.469	NS

SR (all loci)	Na (mean)	H_{Obs} (mean)	H_{Exp} (mean)	PIC (mean)	Combined PID	Combined PID–SIB	Ni	MT
All	5.3	0.519	0.565	0.510	0.00000005	0.0007675	213	0.9522
Germany	5.1	0.535	0.558	0.504	0.00000008	0.0008538	152	0.9563
Greece	4.6	0.478	0.541	0.470	0.00000046	0.0013198	61	0.9419

these markers from the analysis resulted in slight shifts in the overall mean observed and expected heterozygosities to 0.512 and 0.581, respectively; PIC was 0.512.

We found no consistent significant evidence of genotyping error, null alleles, or allelic drop–out in either sample (see table 3). When grouping the two samples together, Cc50 and Cc58 deviated from Hardy–Weinberg equilibrium (HWE), but individually, only the sample from northeast Germany exhibited this deviation (see table 2). Furthermore, no evidence of linkage disequilibrium was found across the two samples or for a single study site across all loci (see table 3). Heightened inbreeding was found in the Greek sample from high F_{IS}, a deficit of heterozygosity, and a high frequency of null alleles compared to the German sample. Overall, there was significant genetic structure between study sites (F_{ST} = 0.04877, *p*–value < 0.001; permutations test, 1,000 permutations; see table 3).

Table 3. Summary of tests of genetic structure, proportion of null alleles, allelic drop–out, inbreeding, and linkage disequilibrium: † For 1,000 permutations (* 0.01 < *p* < 0.05; ** 0.001 < *p* < 0.01; *** *p* < 0.001); †† Based on pairs of retyped individuals (n = 19–38, mean ± SD 28.55 ± 08.02); ††† Bonferroni correction (α/55 = 0.000909091).

Tabla 3. Resumen de las pruebas de estructura genética, alelos nulos, pérdida de alelos, endogamia y desequilibrio de ligamiento: † Para 1.000 permutaciones (0,01 < p < 0,05; ** 0,001 < p < 0,01; *** p < 0,001); †† Basado en pares de individuos que se volvieron a genotipar (n = 19–38, mean ± DE 28.55 ± 08.02); ††† Corrección de Bonferroni (α/55 = 0.000909091).*

	Genetic structure F_{ST}†	Proportion of null alleles		Allelic	Inbreeding F_{IS}†		Linkage disequilibrium†††	
	Ge *vs.* Gr	Ge	Gr	drop–out††	Ge	Gr	Ge	Gr
Cc10	0.02716**	0.00001	0.03724	0.000	–0.00777	0.10261*	NS	NS
Cc15	0.00900	0.04774	0.00324	0.000	0.21122	0.01793	NS	NS
Cc18	0.03098*	0.00002	0.04425	0.000	–0.05765	0.14174	NS	NS
Cc37	0.03179**	0.08457	0.00001	0.000	0.14499*	–0.03082	Cc50, Cc58	NS
Cc42	0.00448	0.00001	0.00000	0.077	–0.02735	–0.09492	NS	NS
Cc44	0.01337	0.00001	0.02865	0.000	–0.06596	0.10757	NS	Cc69
Cc50	0.03346**	0.09735	0.06924	0.000	0.25843***	0.20657*	Cc58, Cc37	NS
Cc58	0.00034	0.05805	0.05039	0.000	0.12153**	0.16632*	Cc50, Cc37	NS
Cc61	0.00405	0.01796	0.05980	0.000	–0.00343	0.16964	NS	NS
Cc69	0.23773***	0.03976	0.11330	0.000	0.07815	0.29346**	NS	Cc44
Cc72	0.01420*	0.00000	0.01632	0.000	–0.07271	0.09580	NS	NS
Overall	0.04877***	–	–	–	0.04036*	0.11760***	–	–

Discussion

We successfully characterized a novel, polymorphic set of microsatellite markers for the white stork. When comparing mean number of alleles per locus (MNA = 5.3) and mean expected heterozygosity (H_{Exp} = 0.565) from this marker discovery with that from the set of markers originally published (MNA = 3.5; H_{Exp}=0.41; Shephard et al., 2009), this new set of markers is more polymorphic, has greater heterozygosity, and thus heightened power in genetic studies. Furthermore, the PIC is considered at least moderately informative (Hildebrand et al., 1992), the P_{ID} is highly informative for population–based and individual–based studies, and $P_{ID–Sib}$ is moderately to highly informative (Waits et al., 2001) and more so when used in conjunction with markers previously reported ($P_{ID–Sib}$ < 0.0001; Feldman et al., in preparation); alone, $P_{ID–SIB}$ for these markers is sufficient for individual–based sibship studies.

Although marker Cc15 and Cc18 have only two alleles and Cc15 has low observed and expected heterozygosities (0.118 and 0.136, respectively), removing these loci from the panel substantially increased the likelihood that two sibling–related individuals would share the same genotype profile by chance (change in $P_{ID–Sib}$ from 0.0007678 to 0.0027647). We thus decided to include them in the final panel due to their utility in individual–based relatedness studies.

Deviations in HWE were not seen in both samples for any markers and in cases where deviation was found, a sample–level explanation was also present (*e.g.*, inbreeding at the given locus was also found). We thus concluded that all the markers are well suited for genetic–based studies and any deviations are likely due to sample site characteristics. The F_{ST} between the German and Greek samples implies that the samples are significantly different, suggesting a weak genetic differentiation between these two regions. This differentiation was not found by Shephard et al. (2013) in comparisons of multiple populations using the previously published panel of markers (overall F_{ST} = 0.005; *p*–value > 0.05). We believe this difference shows the greater sensitivity of the newly developed panel of markers.

This set of markers, either alone or in combination with markers previously tested in this species, allows researchers to distinguish between genotypes at the individual level, thus providing a tool for relatedness studies in addition to more exact population genetics studies. By adding these markers to the white stork molecular tool kit, questions at both the within– and between–population scales related to reproduction,

nest fidelity, migration flyway segregation, and plasticity of philopatry can be much more feasibly and accurately assessed.

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