

## Characterization of microsatellite markers for *Saxicola* species

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**Abstract** We characterized 28 unique African stonechat (*Saxicola torquata*) microsatellite loci. Seventeen loci characterized in 24 unrelated *Saxicola torquata axillaris* individuals sampled at Mount Meru, Tanzania displayed 2–26 alleles per locus and observed heterozygosities ranged from 0.29 to 0.92. Heterozygous females and sequence similarity suggested all 17 loci were autosomal. All markers also successfully amplified in nine different species ranging from Europe to Asia, including three endemic Island species. These microsatellite markers will be useful to assess the genetic diversity of the large and widely distributed genus *Saxicola*, a group comprising 11 recognized species with evidences of cryptic diversification. Several species show a narrow range distribution and are of conservation concern.

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Here, we present a new set of microsatellite markers isolated from a songbird, the African stonechat (*Saxicola torquata axillaris*), which will be useful to assess genetic diversity for conservation efforts in other *Saxicola* species. An Illumina paired-end library was created using 1 µg of genomic DNA extracted from a female *S. t. axillaris* (AR02F) by following the standard protocol of the Sure-Select Library Prep Kit, ILM (Agilent Technologies Inc. Santa Clara, California). DNA sequencing was conducted using a MiSeq Benchtop Sequencer (Illumina Inc., San Diego, California).

Primer sets were designed for 28 unique microsatellite sequences (EMBL accession numbers: HG798924–HG798951) using PRIMER3 v0.4.0 (Rozen and Skaletsky 2000). Blood samples were obtained from 24 unrelated

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**Table 1** Microsatellite loci for the *Saxicola* species: (a) primer sequences and characteristics of 21 autosomal African stonechat (*Saxicola torquata axillaris*) microsatellite loci, (b) cross amplification of 16 *Saxicola torquata axillaris* microsatellite loci for ten *Saxicola* species

Locus	EMBL accession number and clone name	Repeat motif	Primer sequence (5'-3')	T <sub>m</sub> (°C)	n	Exp. allele size (bp)	Obs. allele size range and genotype of AR02F <sup>2</sup> (bp)	N <sub>A</sub>	H <sub>E</sub>	H <sub>O</sub>	pHWE	Est. null allele freq.
(a)	Stor01	(ATCC) <sub>16</sub>	F:[HEX]CTCATCTCTGCTTCCATCTG R:GATCAACTGATGTCATCCATGC	59.96 60.36	24	194	181–209 193, 205	19	0.81	0.85	0.63	0.02
	Stor02	(GT) <sub>10</sub>	F:[6FAM]TGGGCTCAAATTTGAAGTGTG R:CTTTGTGCTGCTGCTTTTTCAC	59.69 59.79	23	198	194–202 198	6	0.64	0.63	0.14	0.01
	Stor03	(ATCC) <sub>13</sub>	F:[HEX]AACTGGAGTCCAGGCATC R:GAGTGGCCTGTGTGGAG	60.06 60.31	22	161	159–183 159, 171	15	0.92	0.84	1.00	-0.05
	Stor04	(ATGTT) <sub>21</sub>	F:[HEX]TCCTAAATGGCACATTGCAC R:GCAAGGTAATTTGTCTTCTTGTG	59.69 59.27	24	199	151–207 191, 197	19	0.85	0.89	0.05	0.02
	Stor05	(AC) <sub>13</sub>	F:[HEX]GCCCACTAGCTGACACAC R:TGTGTTGTGCATCATAGTGTATGG	60.48 60.73	21	199	191–197 195	12	0.29	0.27	1.00	-0.07
	Stor06	(AC) <sub>15</sub>	F:[6FAM]CTTGTGCTCACCCCTGTGG R:TAGAGGCAGCCAACTTCTGT	60.29 60.53	21	156	145–159 151, 153	14	0.71	0.65	0.80	-0.05
	Stor07	(GT) <sub>15</sub>	F:[6FAM]CTGTCTGGGCATGAGAAGG R:TTTGCAGTCAGTCAGTACAAAAGC	59.35 59.62	24	153	139–153 143, 153	8	0.65	0.65	0.28	-0.03
	Stor08	(ATCC) <sub>18</sub>	F:[6-FAM]CACAGCTGCTCTGGGAATC R:ACAAAGGATGGAGGGACAG	59.52 59.90	24	162	220–236 224	9	0.50	0.38	0.27	-0.14
	Stor09	(GT) <sub>13</sub>	F:[HEX]TCGGTGTCTGTTGGTATTGC R:GCAGCTGCCTTTCTGTATGTC	59.57 60.04	19	200	200–224 202, 208	8	0.74	0.77	0.69	0.01
	Stor10	(TAA) <sub>13</sub>	F:[6FAM]TTGAAAAGTTACCCTGTTGTG R:GGTACATTTCTGCTTTCAGATCC	59.04 59.15	23	210	195–221 207, 213	26	0.89	0.85	0.46	-0.03
	Stor11	(GT) <sub>15</sub>	F:[HEX]AGAGTGGCAACTTGTTCTTGG R:TGTACCAGCTCTGCATCAATTG	59.39 59.88	24	143	139–147 139, 143	15	0.71	0.67	0.84	-0.04
	Stor12	(TGA) <sub>18</sub>	F:[HEX]GGCAGTGGCATCTGATTTG R:GGGAGATTCTCAACAGTGCAG	60.22 59.86	24	217	193–239 193	23	0.35	0.29	0.56	-0.09
	Stor13	(TGA) <sub>19</sub>	F:[HEX]TGCTGACAAAAGGAGAAAAGC R:CTGTAATCCGGCAGCACAC	60.52 60.28	23	214	189–215 193, 205	25	0.86	0.86	0.12	-0.01
	Stor14	(GT) <sub>12</sub>	F:[6-FAM]CCATGTAAGGCACTTCCAAAAC R:GGGAGAGGCAGGAACCTGG	60.75 60.76	22	141	138–144 142	8	0.75	0.75	0.42	-0.01
	Stor15	(CT) <sub>11</sub>	F:[6FAM]TCATTAAGGTTTGACTGTGTTGC R:AAGGGCAAGATTCTCTGTTG	59.19 59.17	21	125	120–124 120, 124	7	0.46	0.49	0.05	0.06
	Stor16	(AC) <sub>15</sub>	F:[6FAM]AAACAGAAACAAACCTCCATGTG R:CTCCAGCCTATTGTATCATCACTATC	59.00 59.49	23	201	194–206 200	5	0.60	0.64	0.65	0.02
	Stor17	(CAA) <sub>13</sub>	F:[HEX]AACAGAACTGCTGGCAAACCTG R:TTGATGTTTTCAGCATGGTC	59.09 59.50	22	232	221–237 233	5	0.70	0.76	0.15	0.02

**Table 1** continued

Locus	EMBL accession number and clone name	Repeat motif	Primer sequence (5'-3')	T <sub>m</sub> (°C)	n	Exp. allele size (bp)	Obs. allele range and genotype of AR02F* (bp)	N <sub>A</sub>	H <sub>E</sub>	H <sub>O</sub>	pHWE	Est. null allele freq.	
Stor18*	HG798941	(CTG) <sub>11</sub>	F:[HEX]GAGTCTCAAAGCTTGCCTTC R:TCC TTGAGGAGAGGGTTAAAGG	60.66 60.93	24	155	145–170	7	0.69	0.71	<b>0.02</b>	0.02	
Stor23*	HG798946	(GGAT) <sub>16</sub>	F:[HEX]GCATCCTGAGACCATGTGTG R:CGTCCATACCCATGTCA GTG	60.12 59.83	24	193	191–215 195, 199	8	0.83	0.79	<b>0.01</b>	–0.03	
Stor24*	HG798947	(ATCC) <sub>22</sub>	F:[6FAM]JAGAGCTGAGTCTTCCCAAAG R:CCTGGATCAGGCAAGTGG	60.00 60.20	23	240	238–256	5	0.48	0.90	<b>0.00</b>	0.30	
Stor26*	HG798949	(ATCC) <sub>12</sub>	F:[HEX]AATTCTTCATCCCATTTCCATTATAG R:GAGCTGGGACCACAAAGATTC	59.86 59.66	23	152	137–159	7	0.79	0.76	<b>0.00</b>	0.11	
Locus	<i>S.t. axillaris</i> Kenya (24 tested)		<i>S.t. rubicola</i> Spain (30 tested)			<i>S. caprata</i> Indonesia (7 tested)		<i>S. dacotiae</i> Canary Islands (24 tested)					
	Exp. allele size (bp) ‡	n	Obs. allele size range and genotype of AR02F* (bp)	H <sub>E</sub>	H <sub>O</sub>	pHWE	n	Obs. allele size range (bp)	n	Obs. allele size range (bp)	H <sub>E</sub>	H <sub>O</sub>	pHWE
Stor01	194	24	181–209 193, 205	0.81	0.83	<b>0.03*</b>	6	182–226	19	182–202	0.63	0.74	1.00
Stor02	198	23	194–202 198	0.48	0.46	<b>0.007*</b>	7	190–204	21	192–198	0.18	0.14	0.14
Stor03	161	22	159–183 159, 171	0.73	0.73	0.21	6	159–179	19	155–177	0.82	0.90	0.90
Stor04	199	24	151–207 191, 197	0.88	0.86	0.92	6	133–191	20	137–187	0.87	0.65	<b>0.03*</b>
Stor05	199	21	191–197 195	0.14	0.11	0.11	6	189–195	22	187–193	0.31	0.36	1.00
Stor06	156	21	145–159 151, 153	0.55	0.54	0.05	7	139–153	22	149–153	0.53	0.59	0.84
Stor07	153	24	139–153 143, 153	0.67	0.	0.14	7	144–174	22	148–150	0.30	0.36	1.00
Stor08	162	24	220–236 224	–	–	–	5	224	20	224	–	–	–
Stor09	200	19	200–224 202, 208	0.88	0.88	1.00	5	202–230	20	190–200	0.31	0.20	<b>0.01*</b>
Stor10	210	23	195–221 207, 213	0.18	0.12	0.20	6	186–190	20	190	–	–	–

**Table 1** continued

Locus	<i>S.t. axillaris</i> Kenya (24 tested)			<i>S.t. rubicola</i> Spain (30 tested)			<i>S. caprata</i> Indonesia (7 tested)			<i>S. dacotiae</i> Canary Islands (24 tested)			
	Exp. allele size (bp) ‡	n	Obs. allele size range and genotype of AR02F* (bp)	n	Obs. allele size range (bp)	H <sub>E</sub>	H <sub>O</sub>	pHWE	n	Obs. allele size range (bp)	H <sub>E</sub>	H <sub>O</sub>	pHWE
Stor11	143	24	139–147 139, 143	26	139–173	0.87	0.65	<b>0.005*</b>	7	133–159	0.05	0.05	–
Stor12	217	24	193–239 193	27	181–211	0.87	0.81	0.37	7	177–205	0.09	0.09	1.00
Stor13	214	23	189–215 193, 205	24	184–214	0.88	0.75	<b>0.01*</b>	6	166–224	0.23	0.25	1.00
Stor14	141	22	138–144 142	25	137–159	0.50	0.48	1.00	6	135–159	0.29	0.33	1.00
Stor15	125	21	120–124 120, 124	28	121–135	0.57	0.46	<b>0.001*</b>	7	123–167	0.50	0.41	0.60
Stor16	201	23	194–206 200	27	194–202	0.58	0.52	0.08	7	182–204	0.05	0.05	–

Locus	<i>S. gutturalis</i> Timor-Leste (6 tested)			<i>S. insignis</i> Nepal (1 tested)			<i>S. maura</i> Kazakhstan (4 tested)			<i>S. rubetra</i> Spain (15 tested)			<i>S. sibilla</i> Madagascar (6 tested)			<i>S. tectes</i> Réunion (1 tested)		
	n	Obs. allele size range (bp)	Obs. allele size range (bp)	n	Obs. allele size range (bp)	Obs. allele size range (bp)	n	Obs. allele size range (bp)	H <sub>E</sub>	H <sub>O</sub>	pHWE	n	Obs. allele size range (bp)	H <sub>E</sub>	H <sub>O</sub>	Obs. allele size range (bp)		
Stor01	3	172–226	–	1	198–226	160–226	4	160–226	0.86	0.75	0.64	4	190–218	–	–	206–214		
Stor02	5	190–204	194–196	2	192–196	190–202	8	190–202	0.77	0.50	0.05	6	198–202	–	–	200–202		
Stor03	6	147–175	155–163	2	163	147–187	11	147–187	0.87	0.55	<b>0.03*</b>	6	163–219	–	–	183–191		
Stor04	3	147–207	137–145	2	149–161	131–159	12	131–159	0.88	0.75	<b>0.005*</b>	6	151–217	–	–	193		
Stor05	6	187–195	191–193	3	189–191	177–203	14	177–203	0.70	0.71	0.40	6	195–199	–	–	195		
Stor06	5	143–159	–	3	149–151	139–153	13	139–153	0.89	0.85	0.68	6	145–155	–	–	151–153		
Stor07	6	128–158	150	3	146–156	144–204	12	144–204	0.91	0.67	<b>0.002*</b>	6	144–166	–	–	150–152		
Stor08	4	224	224	2	224	224	11	224	–	–	–	6	224	–	–	224		
Stor09	4	196–218	–	2	190–202	196–220	11	196–220	0.84	0.46	<b>0.0003*</b>	5	200–214	–	–	204–214		
Stor10	5	178–192	186–190	2	190–194	172–196	11	172–196	0.75	0.72	1.00	4	190–210	–	–	190–196		
Stor11	3	133–163	133–163	4	143–163	133–167	14	133–167	0.73	0.71	0.72	6	137–159	–	–	141–145		
Stor12	6	181–207	181–187	3	181–191	177–209	14	177–209	0.87	0.71	<b>0.03*</b>	6	191–235	–	–	205–209		
Stor13	5	192–208	182–198	2	194–208	176–230	9	176–230	0.92	0.89	0.63	3	188–220	–	–	194–196		

**Table 1** continued

Locus	<i>S. gutturalis</i> Timor-Leste (6 tested)		<i>S. insignis</i> Nepal (1 tested)		<i>S. maura</i> Kazakhstan (4 tested)		<i>S. rubetra</i> Spain (15 tested)		<i>S. sibilla</i> Madagascar (6 tested)		<i>S. tectes</i> Réunion (1 tested)	
	n	Obs. allele size range (bp)	Obs. allele size range (bp)	Obs. allele size range (bp)	n	Obs. allele size range (bp)	n	Obs. allele size range (bp)	n	Obs. allele size range (bp)	n	Obs. allele size range (bp)
Stor14	4	153	137–143	155–163	2	137–159	10	137–141	6	137–141	1	137
Stor15	5	129–159	117–129	121–137	3	119–177	12	119–121	6	119–121	1	121
Stor16	6	182–228	196–198	198–210	4	176–209	13	196–204	5	196–204	1	196–200

*S. t. axillaris* *Saxicola torquata axillaris*, *S. t. rubicola* *Saxicola torquata rubicola*, ¥ based on the sequenced *Saxicola torquata axillaris* individual from which primers were designed (AR02F)

*T<sub>m</sub>* melting temperature (°C) of primer, *bp* base pairs, *Exp.* expected, *Obs.* observed allele size, *NA* number of alleles observed, *n* number of individuals genotyped, sex based on adult plumage, *H<sub>o</sub>* observed heterozygosity, *H<sub>E</sub>* expected heterozygosity, *p<sub>HWE</sub>* probability of deviation from Hardy–Weinberg equilibrium, *Est.* estimated null allelic frequency

\* Loci (shown in bold) and taxa, which deviated from Hardy–Weinberg equilibrium (*p* < 0.05):

*Stor18*, *Stor23*, *Stor24*, and *Stor26* or *Saxicola torquata axillaris*

*Stor01*, *Stor02*, *Stor10*, *Stor13* and *Stor15* for *Saxicola torquata rubicola*

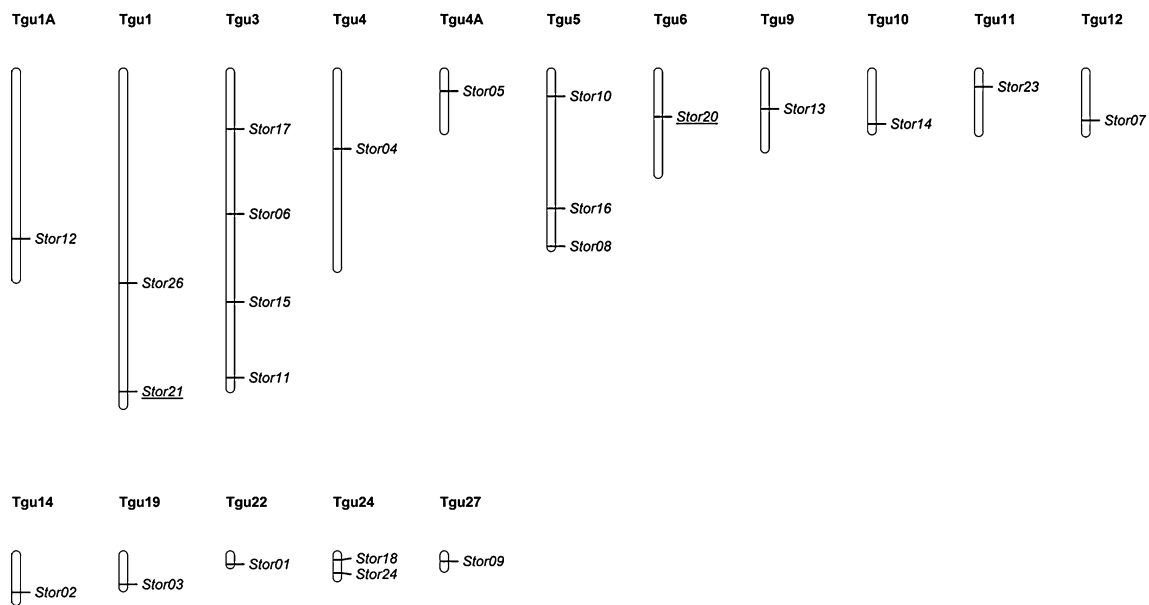
*Stor04* and *Stor09* for *Saxicola dacotiae*

*Stor03*, *Stor04*, *Stor07*, *Stor09* and *Stor12* for *Saxicola rubetra*

adult *S. t. axillaris* individuals sampled at Mount Meru, Arusha National Park, Tanzania. Genomic DNA was extracted using an ammonium acetate precipitation method. Primer sets were amplified individually in all 24 individuals. Each 2-µl PCR contained approximately 10 ng of air-dried genomic DNA, 0.2 µM of each primer and 1 µl QIAGEN PCR mix (QIAGEN Inc.). PCR amplification was performed using a DNA Engine Tetrad<sup>®</sup> Thermal Cycler (MJ Research, Bio-Rad, Hemel Hempstead, Herts, UK) with the following program: 95 °C for 15 min, followed by 35 cycles of 94 °C for 30 s, 58 °C for 90 s, 72 °C for 60 s, 45 cycles of 60 °C for 30 min. Locus-specific products were loaded separately on an ABI 3730 48-well capillary DNA Analyzer (Applied Biosystems, California, USA) and allele sizes assigned using GENEMAPPER v4.1 (Applied Biosystems). The numbers of alleles and heterozygosities were calculated for each locus using CERVUS v3.0.6. Tests for deviation from Hardy–Weinberg equilibrium and linkage disequilibrium were conducted using GENEPOP web version 4.2. To identify sex-linked loci, 318 (76 females, 242 males) *S. t. axillaris* individuals were assessed for heterozygosity (sex based on adult plumage and the Z-002A and Z-002D sex-typing markers; Dawson 2007) and the chromosomal location of each locus was assigned in the zebra finch (*Taeniopygia guttata*) assembled genome by performing a BLAST search for sequence similarity (via <http://www.ensembl.org/index.html>), following Dawson et al. (2006; homologous sequences possessed E-values less than E–05) and a figure created using MAPCHART v2.2.

Of the 28 markers tested, 21 were polymorphic with 2–26 alleles, two were monomorphic, and five amplified multiple non-specific products despite testing at various annealing temperatures (Table 1 and Online Resources 1 and 2). Loci *Stor20* and *Stor21* were monomorphic with the same allele sizes in three other populations (data not shown). None of the 21 polymorphic loci were sex-linked based on the presence of female heterozygotes and sequence similarity to zebra finch autosomes (Fig. 1). Locus *Stor26* displayed a high estimated null allele frequency (over 10 %). No groups of loci exhibited evidence of linkage disequilibrium.

Sixteen selected markers (*Stor01–Stor16*) were assessed in nine other *Saxicola* species, including taxa classified as near threatened (*S. dacotiae* and *S. gutturalis*) or vulnerable (*S. insignis*; Table 1 and Online Resource 1). Between 11 and 16 loci were variable per species (1–24 individuals tested per species). The avian genus *Saxicola* is distributed throughout Africa, Asia, Europe and various islands across Oceania (Illera et al. 2008). Many species within this complex are listed as endangered. Thus, these microsatellites will be useful to assess genetic diversity for conservation efforts.



**Fig. 1** Chromosomal locations of 23 African stonechat (*S. torquata*) loci in the zebra finch *T. guttata* (Tgu) genome. The two monomorphic loci are underlined. Chromosome locations are not assigned for the five loci that amplified multiple non-specific products

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