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Divergence of the Populations of Yellow Wagtail *Motacilla flava*l Linnaeus, 1758, And Citrine Wagtail *Motacilla citreola* Pallas, 1776, (Motacillidae, Passeriformes) in Middle Volga Region of Russia

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Abstract

Blood samples of so-called "yellow" wagtails collected in geographical areas of Middle Volga breeding populations of these species were studied. After mtDNA isolation barcoding of studied species of "yellow" wagtails was performed. Site of gene cytochromec-oxidase I was amplified. This gene was used as a genetic marker for the comparison of obtained samples. After sequencing and sequence alignment of gene cytochrome c-oxidase I, based on the comparison of genetic distances between specimens of the studied species using Jalview software, phylogenetic trees of populations of species *Motacilla flava* L. and *Motacilla citreola* Pall. were constructed.

Keywords: phenotype, genotype, mtDNA barkoding, population, species, birds, "yellow" wagtails, Middle Volga Region of Russia

1. Introduction

Among themost taxonomically controversial groups of passerinebirds polytypical complex *Motacilla flava* in sensu lato (Gladkov, 1954; Portenko, 1960; Stepanyan, 1990; Sotnikov, 2006; Artemieva & Muraviev, 2012b) take saspecial place. An extremely complex individual and geographical variability is in herent to the forms of this group (Zarudny, 1891; Beregovoy, 1970; Bakhtadze, 1987; Grichik, 1992; Babenko, 1981; Red'kin, 2001a, 2001b; Muraviev, Artemieva, Beme, 2014; Cramp, 1988; Artemieva, Muraviev, & Beme, 2013). In addition to the environmental and etological factors of reproductive isolation of sympatric bird sspecies molecular-genetic features of species can play an insulating role too.To identify the real kinship within the taxa an integrated approach that combines assessment of the variability of phenotypic and genotypic features of specific forms including molecular-genetic attributes is required (Artemieva & Muraviev, 2012a; Pavlova et al., 2003; Vili et al., 2009).

Objective: To identify phenotypic and genotypic divergence in populations of yellow wagtail *Motacilla flava* and citrine wagtail *Motacilla citreola* under sympatric conditions in the Middle Volga region of Russia.

2. Materials and Methods

Species of the "yellow" wagtails *Motacilla flava* Linnaeus, 1758, and *Motacilla citreola* Pallas, 1776 (Passeriformes, Motacillidae) subgenus *Budytes* Cuiv. 1817, were selected as the object of molecular-genetic research. We used 11 dried blood samples of *M. flava* on filter paper (6–9.05.2012) and 9 dried blood samples of *M. citreola* on filter paper (5–9.05.2012) collected in the Nizhniy Novgorod (floodplains of Volga and Oka, wastewater treatment plants, meadows).

Samples for genetic studies were collecte dusing the non-invasive sampling method – the birds for blood sample picking were caught with arachnoid ornithological net. Morphometric and plumage characteristics were taken and the bird was ringed. Original value scale for gradation of morphometry parameters as well as plumage color and pattern of birds was developed (Table 1 & 2). System of color and plumage pattern features by Ryabitsev (2001) was applied.

Blood samples were taken from the brachial vein of bird swhen ringing using IsoCode STIXTM paper (special impregnated paper that can be easily used in the field conditions. It significantly reduces the stress effects, as only 1–2 drops equal to 10–12 μl – an amount sufficient to genetic expertise – is taken).

DNA isolation from dried blood samples was carried outon the paper (CosmoBio, Schleicher & Schuell Biosciences) using GeneJET Whole Blood Genomic DNA Purification Mini Kit (Thermo Scientific). Samples were cut out from paper, homogenized and incubated in lysing solution (Lysis Solution, Thermo Scientific) containing proteinase K (56°C, 15 minutes). Further, the DNA was precipitated with 96% ethanol and recovered on silicon columns (GeneJET Genomic DNA Purification Columns, Thermo Scientific).

Fragment of the gene cytochrome C oxidase I (COI) was used as the genetic marker. To amplify the area of interest the following PCR mixture was used (for 20 µl): dNTP (250 µM), primers (0.5 µM), the buffer (1X), taq-polymerase (10 u), DNA template (1 µl), deionized water (to a final volume). Polymerase chain reaction was performed using a thermal cycler FlexCycler (Analytik Jena) with the following temperature settings: DNA denaturation - 94°C, 2 min; 30 cycles under the next conditions: DNA denaturation - 94°C, 30 sec, primer annealing - 55°C, 30 sec., elongation - 72°C, 40 sec.; chain completion - 72°C, 5 min. The results of the reaction were assessed and the fragments were separated in 1% analytical agarose gel;after that preparative gel for the isolation and purification of the fragment of interest (using GeneJET Gel Extraction Kit (Thermo Scientific))was made.

The purified amplified products of the same length were sequenced using capillary genetic analyzer ABI PRISM 3500 (Life Technologies) (with preliminary sequencereaction with fluorescent-labeled deoxyribonucleotides (ddNTP) and subsequent purification of terminated fragment set). The obtained sequences were analyzed and adjusted using Sequence Scanner 2software (Life Technologies Corporation) [http://www.lifetechnologies.com]. The resulting sequence of the gene cytochrome C oxidase I was compared to the same available in GenBank [http://www.ncbi.nlm.nih.gov/genbank] and aligned using the ClustalW2 (EMBL-EBI) software. Genetic distances between specimens were determined using MEGA 4 software (Neighbor-joining method).

3. Results

Morphometry was carried out according to the standard procedures, the basic features of male and female plumage of yellow wagtail *M. flava* and citrine wagtail *M. citreola* were revealed, pairwise correlation analysis of plumage features and morphometry was performed. After that, the most informative features(uncorrelated with each other and with any one of the other parameters) were defined: for *M. flava* – tarsus length, body length, mustache coloration, for *M. citreola* – tarsus length, body length, necklace (Table 1–4).

Table 1. Morphometric parameters of *Motacilla flava*

№	Series,No. of ring	Catch date	Catch time	Sex (P1)	Age (P2)	Fat (P3)	Weight (P4)	Wing length (P5)	Tail length (P6)	Tarsus length (P7)	Cul1 (P8)	Body length (P9)
fl1	XH 51036	6.05	18-20	1 m	1 ad	0	20,0	84,0	68,0	32,0	11,0	16,3
fl2	XH 51039	6.05	19-45	1 m	1 ad	0	17,0	82,0	76,0	30,0	11,0	16,0
fl3	XH 51046	7.05	9-05	1 m	1 ad	3	16,6	81,0	72,0	23,1	9,3	16,7
fl4	XH 51048	7.05	10-30	1 m	1 ad	2	17,1	81,0	76,0	23,5	9,0	17,2
fl5	XH 51051	7.05	20-40	2 f	1 ad	0	15,0	75,0	71,0	23,43	10,0	14,5
fl6	XH 51052	7.05	20-40	1 m	1 ad	0	18,5	83,0	75,0	24,91	11,0	16,1
fl7	XH 51053	8.05	6-45	1 m	1 ad	0	16,5	82,0	71,0	23,3	9,7	16,5
fl8	XH 51058	8.05	10-20	1 m	1 ad	1	17,3	86,0	74,0	23,0	9,5	16,3
fl9	XH 51060	8.05	12-30	1 m	1 ad	0	16,7	83,0	71,0	24,9	9,3	15,4
fl10	XH 51067	9.05	8-10	1 m	1 ad	1	17,5	84,0	68,0	23,8	9,5	15,5
fl11	XH 51073	9.05	21-40	2 f	1 ad	0	16,4	78,0	70,0	23,8	8,7	14,0

Plumage pattern and color features of *Motacilla flava* expressed in points:

- fl1, XH 51036 (series and number of the ring), catch date – 6.05, catch time – 18-20, sex (1 m), subspecies *thunbergi*, white whiskers (P10) – 1 point, dark gray bregma (P11) – 2 points, narrow eyebrow (P12) – 1 point, white chin (P13) – 1 point, dark gray ear coverts (P14) – 2 points, olive-green back (P15) – 2 points, bright yellow breast (P16) – 3 points.
- fl2, XH 51039 (series and number of the ring), catch date– 6.05, catch time– 19-45, sex (1 m), subspecies *thunbergi*, white mustache (P10) – 1 point, dark gray bregma (P11) – 2 points, narrow eyebrow (P12) – 1 point, yellow chin (P13) – 3 points, dark gray ear coverts (P14) – 2 points, olive-green back(P15) – 2 points, bright yellow breast (P16) – 3 points.
- fl3, XH 51046 (series and number of the ring), catch date – 7.05, catch time – 9-05, sex (1 m), subspecies *flava*, white whiskers(P10) – 1 point, gray bregma (P11) – 1 point, wide eye brow (P12) – 2 points, white chin (P13) – 1 point, gray ear coverts (P14) – 1 point, olive-green back(P15) – 2 points, bright yellow breast (P16) – 3 points.
- fl4, XH 51048 (series and number of the ring), catch date– 7.05, catch time– 10-30, sex (1 m), subspecies *flava*, half white whiskers(P10) – 2 points, gray bregma (P11) – 1 point, wide eyebrow (P12) – 2 points, white chin (P13) – 1 point, gray ear coverts (P14) – 1 point, olive-green back (P15) – 2 points, bright yellow breast (P16) – 3 points.
- fl5, XH 51051 (series and number of the ring), catch date – 7.05, catch time – 20-40, sex (2 f), subspecies *flava*, grayish whiskers(P10) – 3 points, gray bregma (P11) – 1 point, wide eyebrow (P12) – 2 points, whitish-yellowish chin (P13) – 2 points, gray ear coverts (P14) – 1 point, olive-grayish back(P15) – 1 point, grayish-yellowish breast (P16) – 1 point.
- fl6, XH 51052 (series and number of the ring), catch date – 7.05, catch time – 20-40, sex (1 m), subspecies *thunbergi*, half white whiskers(P10) – 2 points, dark gray bregma (P11) – 2 points, narrow eyebrow (P12) – 1 point, yellow chin(P13) – 3 points, dark gray ear coverts (P14) – 2 points, olive-green back(P15) – 2 points, bright yellow breast (P16) – 3 points.
- fl7, XH 51053 (series and number of the ring), catch date – 8.05, catch time – 6-45, sex (1 m), subspecies *flava*, white whiskers(P10) – 1 point, gray bregma (P11) – 1 point, wide eyebrow (P12) – 2 points, white chin (P13) – 1 point, gray ear coverts (P14) – 1 point, olive-green back(P15) – 2 points, yellow breast with dark spots (P16) – 2 points.
- fl8, XH 51058 (series and number of the ring), catch date – 8.05, catch time – 10-20, sex (1 m), subspecies *thunbergi*, half white whiskers(P10) – 2 points, dark gray bregma (P11) – 2 points, no eyebrow (P12) – 0 points, white chin (P13) – 1 point, dark gray ear coverts (P14) – 2 points, olive-green back(P15) – 2 points, yellow breast with dark spots (P16) – 2 points.
- fl9, XH 51060 (series and number of the ring), catch date – 8.05, catch time – 12-30, sex (1 m), subspecies *thunbergi*, yellow whiskers(P10) – 4 points, dark gray bregma (P11) – 2 points, narrow eyebrow (P12) – 1 point, yellow chin(P13) – 3 points, dark gray ear coverts (P14) – 2 points, olive-green back(P15) – 2 points, bright yellow breast (P16) – 3 points.
- fl10, XH 51067 (series and number of the ring), catch date – 9.05, catch time – 8-10, sex (1 m), subspecies *flava*, grayish-yellow whiskers(P10) – 5 points, dark gray bregma (P11) – 1 point, wide eyebrow (P12) – 2 points, yellow chin(P13) – 3 points, gray ear coverts (P14) – 1 point, olive-green back(P15) – 2 points, yellow breast with dark spots (P16) – 2 points.
- fl11 (no blood sample), XH 51073 (series and number of the ring), catch time – 9.05, catch time – 21-40, sex(2 f), subspecies *flava*, grayish whiskers(P10) – 3 points, gray bregma (P11) – 1 point, wide eyebrow (P12) – 2 points, whitish-yellowish chin (P13) – 2 points, gray ear coverts (P14) – 1 point, olive-grayish back(P15) – 1 point, grayish-yellowish breast(P16) – 1 point.

Table 2. Morphometric parameters of *Motacilla citreola*

No.	Series and number of the ring	Catch date	Catch time	Sex (P1)	Age (P2)	Fat (P3)	Weight (P4)	Wing length (P5)	Tail length (P6)	Tarsus length (P7)	Cul1 (P8)	Body length (P9)
ct1	XH 47691	5.05	17-20	1 m	1 ad	0	25,0	80,0	65,0	27,0	11,0	15,0
ct2	XH 51041	7.05	6-15	2 f	1 ad	0	15,0	77,0	71,0	23,8	13,9	14,8
ct3	XH 51042	7.05	6-15	2 f	1 ad	0	16,0	79,0	72,5	24,5	9,9	16,0
ct4	XH 51043	7.05	6-15	1 m	1 ad	0	16,0	85,0	75,0	23,56	9,7	16,0
ct5	XH 51044	7.05	6-15	1 m	1 ad	0	16,0	83,0	72,0	23,77	9,5	16,0
ct6	XH 51045	7.05	9-05	2 f	1 ad	0	16,75	79,0	69,0	23,16	10,4	15,5
ct7	XH 51047	7.05	9-50	1 m	1 ad	1	16,1	79,0	72,0	24,6	9,7	16,0
ct8	XH 51049	7.05	10-30	1 m	1 ad	1	15,6	82,0	71,0	24,3	9,9	15,2
ct9	XH 51068 hybrid	9.05	8-10	1 m	1 ad	1	18,3	83,0	73,0	24,8	9,9	16,8
ct10	XH 50736 Second catch2011 г. hybrid	9.05	9-20	2 f	0	1	17,9	76,0	65,0	24,0	10,1	14,9

Plumage pattern and color features of *Motacilla citreola* expressed in points:

- ct1, XH 47691 (series and number of the ring), catch date – 5.05, sex(2 f), subspecies *citreola*, no eyebrow (P10) – 0 points, black wide neck (nape) (P11) – 2 points, lemon yellow bregma (P12) – 4 points, dark gray back (P13) – 3 points, lemon yellow breast (P14) – 4 points, small black spot at the occiput (P15) – 3 points, wide strips on the wing (P16) – 2 points, no necklace (P17) – 0 points, lemon yellow throat (P18) – 5 points.
- ct2, XH 51041 (series and number of the ring), catch date – 7.05, sex (2 f), subspecies *werae*, yellowish eyebrow (P10) – 1 point, no neck (nape) (P11) – 0 points, pale yellowish bregma (P12) – 3 points, light gray back (P13) – 1 point, dirty-gray breast (P14) – 1 point, gray occiput (P15) – 1 point, narrow strips on the wing (P16) – 1 point, no necklace(P17) – 0 points, yellowish throat (P18) – 1 point.
- ct3, XH 51042 (series and number of the ring), catch date – 7.05, sex (2 f), subspecies *citreola*, yellow eye brow(P10) – 2 points, no neck (nape) (P11) – 0 points, gray-yellow bregma (P12) – 2 points, gray back (P13) – 2 points, yellowish breast (P14) – 2 points, dark gray occiput (P15) – 2 points, narrow strips on the wing (P16) – 1 point, necklace(P17) – 1 point, ocher yellow throat (P18) – 3 points.
- ct4, XH 51043 (series and number of the ring), catch date – 7.05, sex (1 m), subspecies *werae*, no eye brow(P10) – 0 points, narrow dark gray neck (nape) (P11) – 2 points, lemon yellow bregma (P12) – 4 points, gray back (P13) – 2 points, pale lemon yellow breast (P14) – 3 points, gray occiput (P15) – 1 point, wide strips on the wing (P16) – 2 points, no necklace (P17) – 0 points, lemon yellow throat (P18) – 5 points.
- ct5, XH 51044 (series and number of the ring), catch date – 7.05, sex (1 m), subspecies *citreola*, no eye brow (P10) – 0 points, black wide neck (nape) (P11) – 2 points, lemon yellow bregma (P12) – 4 points, dark gray back (P13) – 3 points, lemon yellow breast (P14) – 4 points, no black spot at the occiput (P15) – 5 points, wide strips on the wing (P16) – 2 points, necklace (P17) – 1 point, lemon yellow throat (P18) – 5 points.
- ct6, XH 51045 (series and number of the ring), catch date – 7.05, sex(2 f), subspecies *citreola*, yellow eye brow (P10) – 2 points, no neck (nape) (P11) – 0 points, gray-yellow bregma (P12) – 3 points, light gray back(P13) – 1 point, yellowish breast (P14) – 2 points, gray occiput (P15) – 1 point, narrow strips on the wing (P16) – 1 point, no necklace (P17) – 0 points, yellow throat (P18) – 2 points.
- ct7, XH 51047 (series and number of the ring), catch date – 7.05, sex (1 m), subspecies *werae*, no eye brow (P10) – 0 points, narrow dark gray neck (nape) (P11) – 2 points, lemon yellow bregma (P12) – 4 points, gray

back (P13) – 2 points, pale lemon yellow breast (P14) – 3 points, gray occiput (P15) – 1 point, wide strips on the wing (P16) – 2 points, no necklace (P17) – 0 points, lemon yellow throat (P18) – 5 points.

- ct8, XH 51049 (series and number of the ring), catch date– 7.05, sex (1 m), subspecies *werae*, no eyebrow (P10) – 0 points, narrow dark gray neck (nape) (P11) – 2 points, lemon yellow bregma (P12) – 4 points, gray back (P13) – 2 points, lemon yellow breast (P14) – 4 points, big black spot at the occiput (P15) – 4 points, wide strips on the wing (P16) – 2 points, no necklace (P17) – 0 points, lemon yellow throat (P18) – 5 points.
- ct9, XH 51068 (series and number of the ring), гибрид, catch date– 9.05, sex (1 m), subspecies *werae*, no eye brow (P10) – 0 points, narrow dark gray neck (nape) (P11) – 2 points, lemon yellow bregma(P12) – 4 points, gray back (P13) – 2 points, lemon yellow breast (P14) – 4 points, big black spot at the occiput (P15) – 4 points, wide strips on the wing (P16) – 2 points, no necklace (P17) – 0 points, lemon yellow throat (P18) – 5 points.
- ct10, XH 51736 (series and number of the ring), hybrid, second catch 2011 г., catch date– 9.05, sex(2 f), subspecies *citreola*, ocher-yellow eyebrow (P10) – 3 points, no neck (nape) (P11) – 0 points, gray bregma (P12) – 1 point, gray back(P13) – 2 points, yellowish breast (P14) – 2 points, gray occiput (P15) – 1 point, narrow strips on the wing (P16) – 1 point, necklace (P17) – 1 point, ocher yellowish throat (P18) – 4 points.

Table 3. Correlations between morphometry and feather coloration characters of *Motacilla flava*. Marked characters and correlations are significant at p < 0,05; N=11 (casewise deletion of missing data)

Characters	P1	P3	P4	P5	P6	**P7**	P8	**P9**	**P10**	P11	P12	P13	P14	P15	P16
P1	1,000	-0,306	-0,561	**-0,851**	-0,256	-0,237	-0,279	**-0,840**	0,267	-0,430	0,392	0,048	-0,430	**-1,000**	**-0,800**
P3	-0,306	1,000	-0,093	0,093	0,202	-0,397	-0,449	0,539	-0,138	-0,407	0,258	-0,450	-0,407	0,306	0,383
P4	-0,561	-0,093	1,000	**0,686**	-0,122	**0,644**	0,540	0,420	-0,247	0,567	-0,448	-0,054	0,567	0,561	0,456
P5	**-0,851**	0,093	**0,686**	1,000	0,057	0,248	0,224	0,572	-0,078	0,591	**-0,653**	-0,010	0,591	**0,851**	0,537
P6	-0,256	0,202	-0,122	0,057	1,000	-0,088	0,150	0,439	-0,409	0,264	-0,301	0,037	0,264	0,256	0,263
P7	-0,237	-0,397	**0,644**	0,248	-0,088	1,000	**0,739**	0,105	-0,371	0,598	-0,355	0,132	0,598	0,237	0,464
P8	-0,279	-0,449	0,540	0,224	0,150	**0,739**	1,000	0,178	-0,416	**0,625**	-0,419	0,270	**0,625**	0,279	0,221
P9	**-0,840**	0,539	0,420	0,572	0,439	0,105	0,178	1,000	-0,569	0,158	-0,186	-0,461	0,158	**0,840**	**0,738**
P10	0,267	-0,138	-0,247	-0,078	-0,409	-0,371	-0,416	-0,569	1,000	-0,194	0,177	0,571	-0,194	-0,267	-0,360
P11	-0,430	-0,407	0,567	0,591	0,264	0,598	**0,625**	0,158	-0,194	1,000	**-0,911**	0,295	**1,000**	0,430	0,399
P12	0,392	0,258	-0,448	**-0,653**	-0,301	-0,355	-0,419	-0,186	0,177	**-0,911**	1,000	-0,084	**-0,911**	-0,392	-0,252
P13	0,048	-0,450	-0,054	-0,010	0,037	0,132	0,270	-0,461	0,571	0,295	-0,084	1,000	0,295	-0,048	-0,098
P14	-0,430	-0,407	0,567	0,591	0,264	0,598	**0,625**	0,158	-0,194	**1,000**	**-0,911**	0,295	1,000	0,430	0,399
P15	**-1,000**	0,306	0,561	**0,851**	0,256	0,237	0,279	**0,840**	-0,267	0,430	-0,392	-0,048	0,430	1,000	**0,800**
P16	**-0,800**	0,383	0,456	0,537	0,263	0,464	0,221	**0,738**	-0,360	0,399	-0,252	-0,098	0,399	**0,800**	1,000

Table 4. Correlations between morphometry and feather coloration characters of *Motacilla citreola*. Marked *correlations are significant at p < 0,01; N=10 (casewise deletion of missing data)

Characters	P1	P2	P3	P4	P5	P6	**P7**	P8	**P9**	P10	P11	P12	P13	P14	P15	P16	**P17**	P18
P2	-0,408	1,000	-0,408	-0,077	0,526	0,590	0,115	0,081	0,392	-0,680	0,408	0,680	0,000	0,287	0,291	0,408	-0,509	-0,000
P3	-0,250	-0,408	1,000	-0,086	-0,090	-0,078	0,061	-0,330	0,140	-0,037	0,250	0,037	0,000	0,273	0,109	0,250	-0,089	0,433
P4	-0,253	-0,077	-0,086	1,000	-0,042	-0,655	**0,865**	0,009	-0,196	-0,132	0,253	0,132	0,531	0,401	0,177	0,253	-0,150	0,321
P5	**-0,764**	0,526	-0,090	1,000	1,000	0,631	-0,021	-0,499	0,627	-0,729	**0,764**	0,729	0,406	0,714	0,546	**0,764**	-0,232	0,623
P6	-0,306	0,590	-0,078	0,631	0,631	1,000	-0,437	-0,205	0,704	-0,456	0,306	0,456	-0,151	0,093	0,114	0,306	-0,149	0,101
P7	-0,392	0,115	0,061	-0,021	-0,021	-0,437	1,000	0,031	-0,099	-0,339	0,392	0,339	0,599	0,472	0,290	0,392	-0,168	0,408
P8	0,446	0,081	-0,330	-0,499	-0,499	-0,205	0,031	1,000	-0,592	0,120	-0,446	-0,120	-0,486	-0,581	-0,321	-0,446	-0,301	-0,727
P9	-0,427	0,392	0,140	0,627	0,627	0,704	-0,099	-0,592	1,000	-0,373	0,427	0,373	0,181	0,410	0,323	0,427	0,014	0,416
P10	**0,909**	-0,680	-0,037	-0,729	-0,729	-0,456	-0,339	0,120	-0,373	1,000	**-0,909**	**-1,000**	-0,440	-0,729	-0,524	**-0,909**	0,526	-0,590
P11	1,000	0,408	0,250	**0,764**	**0,764**	0,306	0,392	-0,446	0,427	**-0,909**	1,000	**0,909**	0,645	**0,899**	0,576	1,000	-0,356	**0,866**
P12	**-0,909**	0,680	0,037	0,729	0,729	0,456	0,339	-0,120	0,373	-1,000	**0,909**	1,000	0,440	0,729	0,524	**0,909**	-0,526	0,590
P13	-0,645	0,000	0,000	0,406	0,406	-0,151	0,599	-0,486	0,181	-0,440	0,645	0,440	1,000	0,757	0,638	0,645	0,345	**0,782**
P14	**-0,899**	0,287	0,273	0,714	0,714	0,093	0,472	-0,581	0,410	-0,729	**0,899**	0,729	0,757	1,000	**0,792**	**0,899**	-0,146	**0,880**
P15	-0,576	0,291	0,109	0,546	0,546	0,114	0,290	-0,321	0,323	-0,524	0,576	0,524	0,638	**0,792**	1,000	0,576	0,161	0,523
P16	1,000	0,408	0,250	**0,764**	**0,764**	0,306	0,392	-0,446	0,427	**-0,909**	1,000	**0,909**	0,645	**0,899**	0,576	1,000	-0,356	**0,866**
P17	0,356	-0,509	-0,089	-0,232	-0,232	-0,149	-0,168	-0,300	0,014	0,526	-0,356	-0,526	0,345	-0,146	0,161	-0,356	1,000	0,000
P18	**-0,866**	0,000	0,433	0,623	0,623	0,101	0,408	-0,727	0,416	-0,590	**0,866**	0,590	**0,782**	**0,880**	0,523	**0,866**	0,000	1,000

On the base of the data obtained by the pairwise correlation analysis of morphometry and plumage features, the tree diagram of studied parameters was built using Ward cluster analysis and their clusters were allocated (Figure 1).

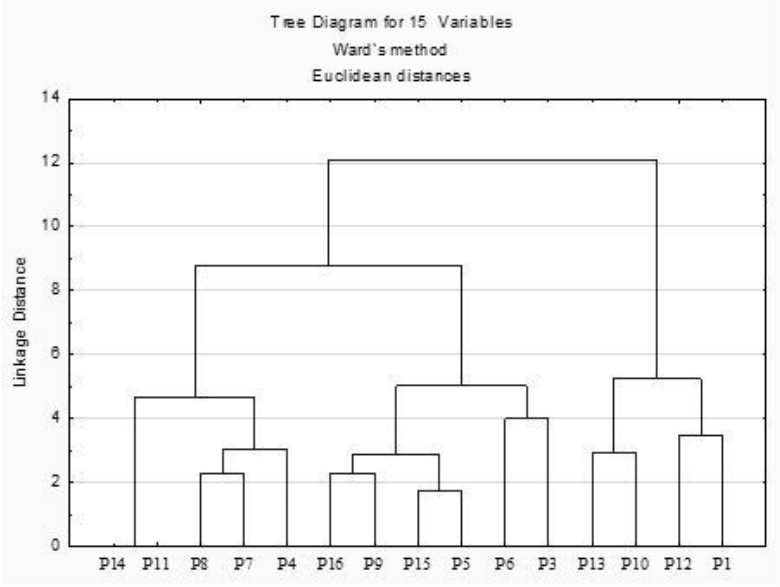

Figure 1. Clusters of morphometry and plumage parameters of *Motacilla flava* obtained with Ward method

On the base of the data obtained by the pairwise correlation analysis of morphometry and plumage features, the tree diagram of studied parameters was built using Ward cluster analysis and their clusters were allocated (Figure 2).

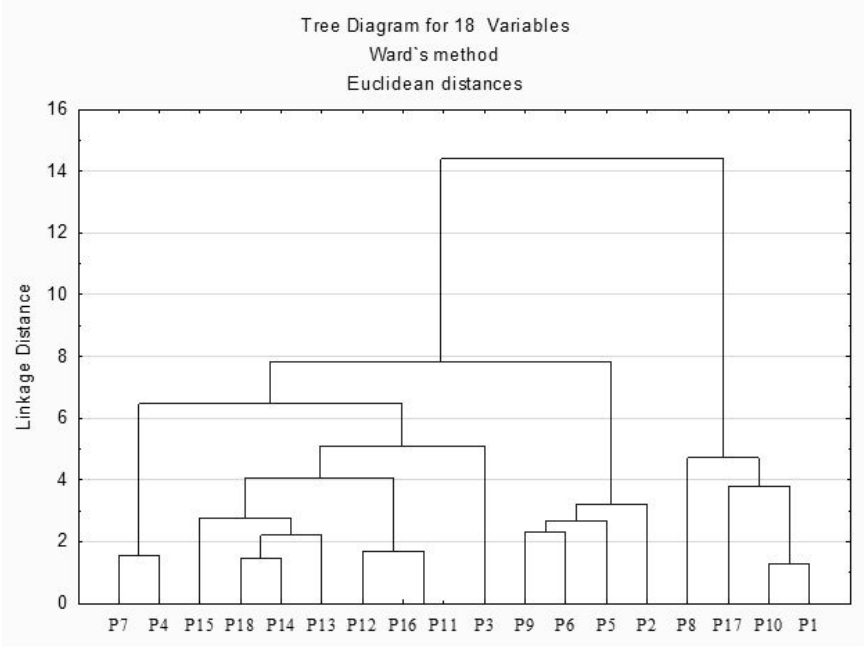

Figure 2. Clusters of morphometry and plumage parameters of *Motacilla citreola* obtained with Ward method

After clustering of the results of the pairwise correlation analysis of morphometry and plumage features by Ward three clusters of samples of yellow wagtail *M. flava* associated with presence of the specimens of subspecies *M. f. flava* and *M. f. thunbergi* in the samples were identified. One specimen turned out to be a hybrid, as well as three

clusters of samples of citrine wagtail *M. citreola* associated with the presence of the specimens of subspecies *M. c. citreola* and *M. c. werae* in the samples. Two specimens were hybrid (Figure 3, 4).

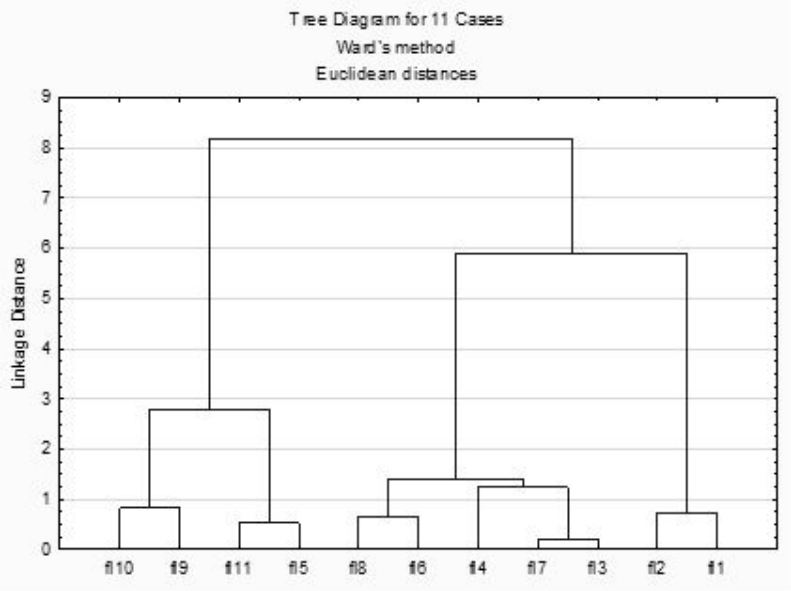

Figure 3. Clusters of *Motacilla flava* in the space of informative parameters obtained by Ward method

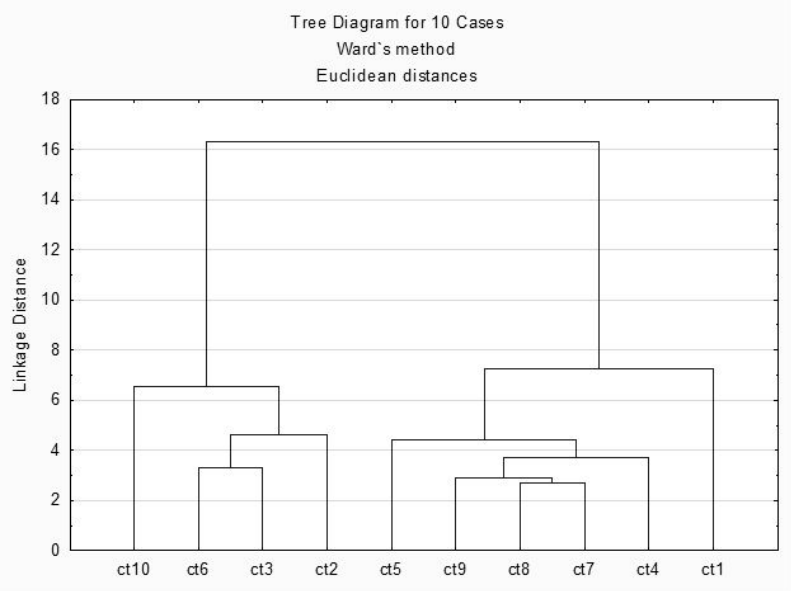

Figure 4. Clusters of *Motacilla citreola* in the space of informative parameters obtained by Ward method

When sequencing the amplified DNA fragments of *M. flava* blood samples sequences of the gene cytochrome c oxidase I were obtained. Phylogenetic trees were constructed using Neighbor-joining method. The sequences of the gene of interest turned out to be different in all dry samples taken from individual birds suggesting genetic heterogeneity of *M. flava* and *M. citreola* populations of wagtails in the studied area. The sequences were aligned using ClustalW2 software, then phylogenetic trees for all specimens of *M. flava* and *M. citreola* populations were constructed using JalView software and genetic distances were defined (Table 5 and 6; Figure 5, 6).

Table 5. Genetic distances between studied specimens of *Motacilla flava* samples in Middle Volga populations obtained with MEGA 4 software

Number of the ring	XH 51067	XH 51060	XH 51058	XH 51053	XH 51052	XH 51051	XH 51048	XH 51046	XH 51039	XH 51036	*XH 50736*
XH 51067	-	0.009	0.009	0.009	0.009	0.009	0.009	0.003	0.009	0.009	*0.014*
XH 51060	0.009	-	0.000	0.000	0.000	0.000	0.000	0.006	0.000	0.000	*0.006*
XH 51058	0.009	0.000	-	0.000	0.000	0.000	0.000	0.006	0.000	0.000	*0.006*
XH 51053	0.009	0.000	0.000	-	0.000	0.000	0.000	0.006	0.000	0.000	*0.006*
XH 51052	0.009	0.000	0.000	0.000	-	0.000	0.000	0.006	0.000	0.000	*0.006*
XH 51051	0.009	0.000	0.000	0.000	0.000	-	0.000	0.006	0.000	0.000	*0.006*
XH 51048	0.009	0.000	0.000	0.000	0.000	0.000	-	0.006	0.000	0.000	*0.006*
XH 51046	0.003	0.006	0.006	0.006	0.006	0.006	0.006	-	0.006	0.006	*0.011*
XH 51039	0.009	0.000	0.000	0.000	0.000	0.000	0.000	0.006	-	0.000	*0.006*
XH 51036	0.009	0.000	0.000	0.000	0.000	0.000	0.000	0.006	0.000	-	*0.006*
XH 50736	*0.014*	*0.006*	*0.006*	*0.006*	*0.006*	*0.006*	*0.006*	*0.011*	*0.006*	*0.006*	-

Table 6. Genetic distances between studied specimens of *Motacilla citreola* samples in MiddleVolga populations obtained with MEGA 4 software

Number of the ring	XH 51047	XH 51049	XH 51041	XH 51068	XH 51045	XH 51042	XH 47691	XH 51043	XH 51044
XH 51047	-	0.011	0.007	0.007	0.011	0.004	0.004	0.004	0.011
XH 51049	0.011	-	0.004	0.004	0.015	0.007	0.007	0.007	0.015
XH 51041	0.007	0.004	-	0.000	0.011	0.004	0.004	0.004	0.011
XH 51068	0.007	0.004	0.000	-	0.011	0.004	0.004	0.004	0.011
XH 51045	0.011	0.015	0.011	0.011	-	0.007	0.007	0.007	0.015
XH 51042	0.004	0.007	0.004	0.004	0.007	-	0.000	0.000	0.007
XH 47691	0.004	0.007	0.004	0.004	0.007	0.000	-	0.000	0.007
XH 51043	0.004	0.007	0.004	0.004	0.007	0.000	0.000	-	0.007
XH 51044	0.011	0.015	0.011	0.011	0.015	0.007	0.007	0.007	-

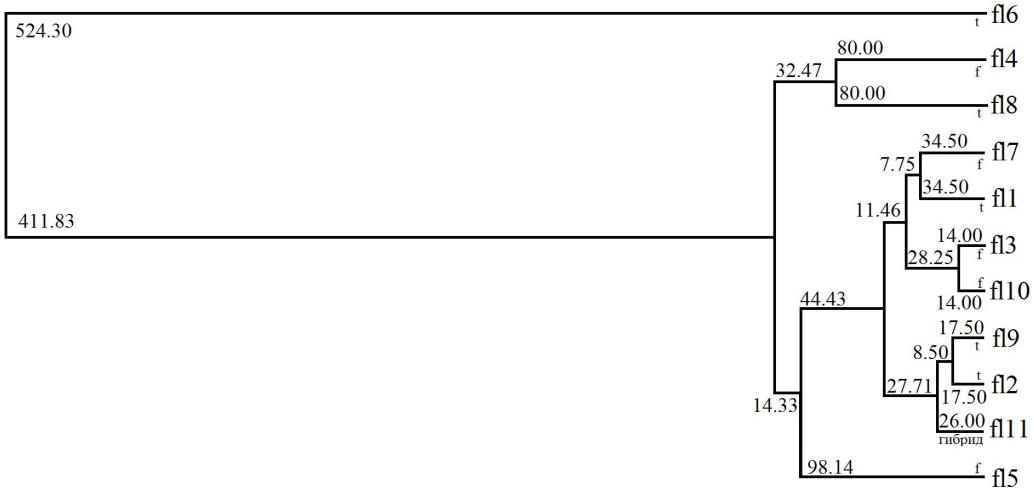

Figure 5. Phylogenetic tree of *Motacilla flava* specimens constructed on the base of genetic analysis of sequences of the gene cytochrome c oxidase I using JalView software, Average Distance method (weighted average)

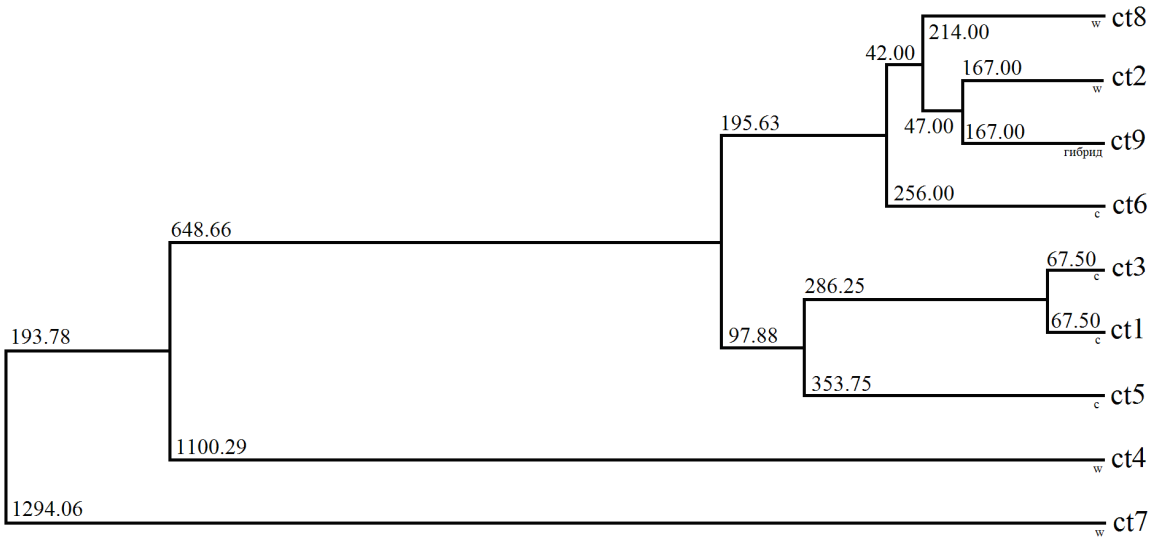

Figure 6. Phylogenetic tree of *Motacilla citreola* specimens constructed on the base of genetic analysis of sequences of the gene cytochrome c oxidase I using JalView software, Average Distance method (weighted average)

4. Discussion

During phylogeographic analysis of nucleotide sequences of mitochondrial genes in the Middle Volga populations of traditionally recognized species *M. flava* and *M. citreola* the lines common in the European part of Russia and neighboring countries which respectively meet subspecies *M. f. flava* and *M. f. thunbergi* were revealed; the third line is related to presence of the hybrid specimens in the studied populations. Within the species *M. citreola* three lines were also revealed corresponding *M. c. citreola* and *M. c. werae* subspecies. This third line also corresponds to the presence of hybrid specimens in the Middle Volga populations.

4.1 Phenotypic and genotypic structure of M. flava populations

Coloration of the "whiskers" (the most informative indication of plumage color) of the specimens of the *M. flava* subspecies varies from yellow to white in the *M. f. thunbergi* and from gray to yellow-gray in *M. f. flava*. According to the genetic research five specimens belong to the *M. f. thunbergi* subspecies, 6 - to the *M. f. flava* subspecies (5 *thunbergi*: 6 *flava*). In the subspecies *M. f. thunbergi* phenotypic splitting of "whiskers" plumage had the next form: 1 specimen with yellow "whiskers", 2 specimens with half yellow (half white) "whiskers", 2 specimens with white "whiskers". In the subspecies *M. f. thunbergi* phenotypic splitting of "whiskers" plumage had the next form: 1 specimen with gray yellow "whiskers", 2 specimens with gray "whiskers", 1specimen with half white "whiskers" and 2 specimens with white "whiskers". The overall ratio of phenotypes: - 4 white : 3 half yellow (half white): 2 gray : 1 yellow : 1 gray yellow. Probably, this feature can be inherited by the complementarity or incomplete dominance type.

4.2 Phenotypic and genotypic structure of M. citreola populations

The presence of "necklace" (the most informative indication of plumage color) in the *M. citreola* subspecies varies discretely – specimens of the *M. c. citreola* subspecies have necklace and specimens of the *M. c. werae* subspecies have not. Hybrid specimens also have two similar kinds of phenotype by the presence of "necklace". In the performed molecular genetic study of 10 *M. citreola* specimens 4 specimens belonged to the subspecies *M. c. werae*, 4 specimens – to subspecies *M. c. citreola* (4 *werae*: 4 *citreola*), two specimens turned out to be hybrid. In the subspecies *M. c. citreola* phenotypic splitting of "necklace" had the next form: 2 specimens had a necklace and 2 specimens hadn't. 4 specimens of the subspecies *M. c. werae* had no "necklace". Phenotype ratio for a given parameter was 7: 3 (2: 1). Probably, this feature can be inherited as following test cross: allele Aa (no "necklace") – *M. c. werae* genotype, AA allele ("necklace") – *M. c. citreola* genotype. Then, when crossed the next splitting will be: 2 Aa (*werae*): 1 aa (*citreola*).

Nevertheless, these subspecies rather differ in some peculiarities of their biology, ecology and morphology. So we can consider these forms at the level of species as evidenced by the comparative analysis of mitochondrial DNA. Defined genotype and phenotype ratios of the area space can mark the area of hybridization of studied phenotypes.

5. Conclusions

Genetic structure of Middle Volga populations of “yellow” wagtails *M. flava* and *M. citreola* is heterogeneous. Subpopulations with prevailing subspecies *M. f. flava* and *M. f. thunbergi* or subspecies *M. c. citreola* and *M. c. werae* were revealed. Also hybrid specimens were observed in these populations. At that *M. f. flava* and *M. f. thunbergi* as well as *M. c. citreola* and *M. c. werae* are well genetically differed with maximum genetic distances equal to 524,30 and 1100,29, 1294,06 respectively. These distances correspond to subspecies level of differences in *M. flava* and to species level in *M. citreola.* Themost in formative parameters of morphometry and plumage color were revealed: tarsus length, body length and “whisker” coloration in *M. flava*and also tarsus length, body length and necklace besides in *M. citreola*.

Subspecies forms of *M. flava* viz. *M. f. flava*, *M. f. thunbergi* are included in the Western complex of *M. flava* forms; subspecies forms of *M. citreola* viz. *M. c. citreola, M. c. werae* form a separate genetic branch of the polytypical group *M. flava*s.1. [11]. In the North-Western and Northern Europe form *M. f. thunbergi*is common where mixed populations of *M. f. flava* and *M. f. thunbergi* nest. Throughout its area males of *M. f. thunbergi*form live sympatrically with white-brow form of *M. f. flava* generating all variants of transitions between these forms by hybridization. Spectrum of autogenetic processes in *M. flava* and *M. citreola* populations in area space in wide sympatric conditions reflects the mechanisms of reproductive isolation of forms of species and subspecies rank. It is the result of microevolution of *M. flava* polytypic complex.

Thus, the possibility of genetic separation between sympatric breeding populations of yellow wagtail *Motacilla flava* and citrine wagtail *Motacilla citreola* in the Middle Volga was revealed.

Phylogeographical analysis of the nucleotide sequences of the mitochondrial gene cytochrome oxidase I in studied populations of "yellow" wagtails revealed the existence of separate lines common in the European part of Russia and neighboring countries including subspecies *M. f. flava, M. f. thunbergi* and *M. c. citreola, M. c. werae* respectively.

So forms *M. c. citreola* and *M. c. werae* need assigning them the status of species due to significant genetic distances.

Despite widespread sympatry in nesting habitats there is a selective mating between males and females of each studied species that prevents free crossing and supports isolating mechanisms in the populations.

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