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Applying integrative taxonomy as a tool for revealing hybridisation events in *Stipa* (Poaceae)

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Abstract. Hybridisation in the wild between closely related species is a common mechanism of speciation in the plant kingdom and, in particular, in the grass family. Here we show the usefulness of integrative taxonomy as a tool for revealing hybridisation events in *Stipa* (one of the largest genera in Poaceae). We demonstrate the usage of the classical morphological approach combining with scanning electron microscopy data, as well as analyses of pollen grains, and the application of molecular techniques including data derived from next generation sequencing. We illustrate the application of this integrative tool by specific examples: (1) in the *S. × heptapotamica* hybrid complex comprising morphologically distant but genetically closely related species, and (2) in the *S. × lazkovii* hybrid complex containing genetically distant species.

Key words: natural hybridisation, ISSR, NGS, DArTseq, pollen viability, SEM, integrative taxonomy, *Stipa*.

Применение интегративной таксономии как инструмента для выявления событий гибридизации в роде *Stipa* (Poaceae)

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Аннотация. Гибридизация в дикой природе между близкородственными видами – распространенный механизм видообразования в царстве растений и, в частности, в семействе злаковых. Здесь мы показываем использование интегративной таксономии как инструмента для выявления событий гибридизации у *Stipa*, одного из крупнейших родов Poaceae. Показано использование классического морфологического подхода в сочетании с данными сканирующей электронной микроскопии, анализом пыльцевых зерен и применением молекулярных методов, включая данные, полученные в результате «секвенирования следующего поколения» (NGS). Применение интегративной таксономии показано на конкретных примерах: (1) в гибридном комплексе *S. × heptapotamica*, включающем морфологически далекие, но генетически близкие виды, и (2) в гибридном комплексе *S. × lazkovii*, содержащем генетически далекие виды.

Ключевые слова: естественная гибридизация, ISSR, NGS, DArTseq, жизнеспособность пыльцы, интрогрессия, SEM, интегративная таксономия, *Stipa*.

Hybridisation in the wild between closely related species is a common mechanism of speciation in the plant kingdom (Goulet et al., 2017). Due to the prevalence of polyploidy found in angiosperms, it has been estimated that around 11 % of flowering plants may have arisen through hybridisation events (Arnold, 2006). In general, hybridisation is often accompanied by introgression and causes gene transfer between species via repeated backcrossing (Suarez-Gonzalez, 2018). On the one hand, it may have contributed to species diversity and speciation, on the other, deleterious consequences of hybridisation such as decreased fitness, genetic assimilation and gene swamping may drive populations toward the brink of extinction (Mota, 2019).

The genus *Stipa* L. belongs to the subfamily Pooideae and alongside with Bambusoideae (bamboos), and Oryzoideae (rices) form the so-called BOP clade. The BOP species are known as the “cool season” or “poid” grasses and all are C₃ and distributed in temperate climates. Following Tzvelev (1974), the genus *Stipa* includes six main sections *Barbatae* Junge, *Leiostipa* Dumort, *Pseudoptilagrostis* Tzvelev, *Regelia* Tzvelev, *Stipa*, and *Smirnovia* Tzvelev, and comprises over 150 species native to Asia, Europe and North Africa (Nobis, 2014; Nobis et al., 2020). In its strict sense, the genus is monophyletic, but subdivisions within the genus are not consistently supported by available molecular data (Kellogg, 2015).

For decades it has been hypothesised that some *Stipa* taxa arose via hybridisation (Smirnov, 1970; Tzvelev, 1976; Kotukhov, 2002; Nobis, 2013). According to our observations, *Stipa* hybrids reproduce vegetatively and, less frequently, sexually (Nobis, 2013). Based on morphology, a hybrid origin can be attributed to ca. 30 % of *Stipa* species, where only in Middle Asia 23 of 72 species are regarded as nothospecies (Nobis et al., 2020).

In the present study, we demonstrate the usefulness of integrative taxonomy as a tool for revealing hybridisation events in *Stipa* by specific examples: (1) in the hybrid complex *S. × heptapotamica* comprising morphologically distant but genetically closely related species, and (2) in the hybrid complex *S. × lazkovii* containing genetically distant species.

The *S. × heptapotamica* hybrid complex includes a very rare and endemic species, *S. heptapotamica* Golosk. that always grows within the contact zones of *S. richteriana* Kar. & Kir. and *S. lessingiana* Trin. & Rupr. in eastern and southern Kazakhstan (Nobis et al., 2019). We hypothesised that *S. heptapotamica* could be a hybrid of these two taxa, which, according to Tzvelev (1974), belong to two different sections, sect. *Leiostipa* Dumort. and sect. *Subbarbatae* Tzvelev, respectively.

To verify our hypothesis, we applied the classical morphometry using the measurement of the 14 most informative morphological characters commonly used in keys and taxonomic description of *Stipa*. In addition, we performed an ultrastructural analysis of the lemma and lamina using scanning electron microscopy (SEM). Both analyses demonstrated that *S. heptapotamica* has intermediate characters between *S. richteriana* and *S. lessingiana*. Further, we accomplished a viability test using Alexander's stain. The test showed that the frequency of viable pollen grains was very high in *S. lessingiana* and *S. richteriana* (94 % and 87 % respectively), while for *S. heptapotamica* it was less than 50 % (Figure 1).

Figure 1. Pollen grains of *Stipa* taxa stained with Alexander's dye: a – *S. lessingiana*, b – *S. heptapotamica*, c – *S. richteriana*. Scale bar 50 μ m. The figure adapted from Nobis et al., 2019

Furthermore, to explore the differences at the molecular level, we performed analyses based on inter simple sequence repeat markers (ISSR) and next-generation sequencing (NGS) data derived from whole chloroplast genomes and the nuclear Intergenic Spacer (IGS). The ISSR markers revealed hybridisation events between *S. lessingiana* and *S. richteriana*, and demonstrated possible introgression processes between *S. lessingiana* and *S. heptapotamica*, whereas NGS data analyses confirmed the maternal inheritance of a plastome from *S. lessingiana* in *S. heptapotamica* and supported the finding of cryptic lineages within *S. richteriana*.

The *S. × lazkovii* hybrid complex includes a very rare and endemic species, *S. × lazkovii* M. Nobis & A. Nowak, that was found in eastern Kyrgyzstan only in one locality (Baiakhmetov et al., 2020). Due to all specimens of *S. × lazkovii* seeming to be morphologically intermediate between two species from the same locality, we hypothesised that they can be hybrids between *S. krylovii* Roshev. and *S. bungeana* Trin. Although, traditionally both putative parental taxa were assigned to the section *Leiostipa* (Tzvelev, 1976), they are distant phylogenetically and belong to two different clades (Nobis et al., 2019; Krawczyk et al., 2017).

For this hybrid complex, we performed an analysis of macro-characteristics based on the 22 most informative quantitative and three qualitative morphological characters. Next, we used an ultrastructural analysis of the lemma and lamina using SEM. Both analyses suggested that *S. × lazkovii* has intermediate characters between *S. krylovii* and *S. bungeana*. Further, we applied the DArTseq approach representing a combination of DArT complexity reduction methods and next generation sequencing platforms (Sansaloni C. et al., 2011). In total, 76,604 silicoDArT and 19,133 SNPs markers revealed that all *S. × lazkovii* specimens are F1 hybrids. In addition, molecular markers detected an introgression event between *S. bungeana* and *S. glareosa* (Figure 2).

The study of the *S. × lazkovii* hybrid complex highlighted the applicability of genome reduction approaches such as DArTseq in studies on natural hybridisation in the wild, and specifically in a grass genus *Stipa*. The high density genome wide genotyping-by-sequencing resulted in a total of 137,437 silicoDArT and 125,850 SNPs markers, of which 76,604 silicoDArT and 19,133 SNPs provided robust information of the *Stipa* genome in the absence of the reference sequence information. Such number of markers is several 100-fold higher than was achieved in the study on the *S. × heptapotamica* hybrid complex.

Figure 2. FastSTRUCTURE result based on 76,604 SilicoDART markers for K = 5
The figure adapted from Baiakhmetov et al., 2020

Thus, currently, in our studies on hybridisation in *Stipa* within integrative taxonomy we successfully apply: (1) classical morphometry, (2) SEM, (3) pollen viability tests, (4) molecular markers including ISSR, Silico DART, as well as SNPs derived from DARTseq, and NGS data of cpDNA and nDNA loci.

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