Genetic structure of the threatened West-Pannonian population of Great Bustard (*Otis tarda*)

Jose L. Horreo, Rainer Raab, Péter Spakovszky, Juan Carlos Alonso

The genetic diversity, population structure and gene flow of the Great Bustards (*Otis tarda*) living in Austria-Slovakia-West Hungary (West-Pannonian region), one of the few populations of this globally threatened species that survives across the Palaearctic, has been assessed for first time in this study. Fourteen recently developed microsatellite loci identified one single population in the study area, with high values of genetic diversity and gene flow between two different genetic subunits. One of these subunits (Heideboden) was recognized as a priority for conservation, as it could be crucial to maintain connectivity with the central Hungarian population and thus contribute to keep contemporary genetic diversity. Current conservation efforts have been successful in saving this threatened population from extinction two decades ago, and should continue to guarantee its future survival.

1   **Genetic structure of the threatened West-Pannonian population of Great Bustard (*Otis***

2   ***tarda*)**

3   Jose Luis Horreo[1], Rainer Raab[2], Péter Spakovszky[2,3], Juan Carlos Alonso[4]

5   [1] Department of Ecology and Evolution, Biophore, University of Lausanne, Lausanne 1015,

6   Switzerland.

7   [2] Technisches Büro für Biologie, Quadenstraße 13, 2232 Deutsch-Wagram, Austria

8   [3] Institute of Wildlife Management and Vertebrate Zoology, University of West Hungary, 9400

9   Sopron, Ady E. u. 5, Hungary

10   [4] Museo Nacional de Ciencias Naturales, Consejo Superior de Investigaciones Científicas

11   (CSIC), José Gutiérrez Abascal 2, Madrid 28006, Spain.

13   Corresponding author: Jose L. Horreo; phone +4121 692 4176; e-mail: horreojose@gmail.com

15   **ABSTRACT**

16   The genetic diversity, population structure and gene flow of the Great Bustards (*Otis tarda*)

17   living in Austria-Slovakia-West Hungary (West-Pannonian region), one of the few populations

18   of this globally threatened species that survives across the Palaearctic, has been assessed for first

19   time in this study. Fourteen recently developed microsatellite loci identified one single

20   population in the study area, with high values of genetic diversity and gene flow between two

21   different genetic subunits. One of these subunits (Heideboden) was recognized as a priority for

22   conservation, as it could be crucial to maintain connectivity with the central Hungarian

23   population and thus contribute to keep contemporary genetic diversity. Current conservation

24   efforts have been successful in saving this threatened population from extinction two decades

25   ago, and should continue to guarantee its future survival.

27   **INTRODUCTION**

28   The Great Bustard (*Otis tarda*, Linnaeus 1758) is a globally endangered species, classified as

29   vulnerable in the Red List of Threatened Species (IUCN 2015). Today the species' stronghold is

30   found in the Iberian Peninsula, with ca. 70% of the world total abundance. Other central

31   European and Asian populations are much smaller and show a fragmented distribution pattern

32   (Alonso & Palacín 2010). The German population and the West-Pannonian population living in

33   Austria-Slovakia-West Hungary suffered particularly dramatic decreases, respectively from

34   4,100 birds in 1939 to 65 in 1995, and from ca. 3,500 birds in 1900 to 130 birds in 1996 (Raab et

35   al. 2010; http://www.grosstrappe.at; http://www.grosstrappe.de). However, both populations

36   survived and have even slightly increased in numbers, reaching nowadays 197 birds in Germany

37   and 505 birds in the West-Pannonian population (February 2015 in both cases), thanks to

38   continued and intensive conservation efforts, centered on habitat management programs in both

39   countries plus captive breeding in Germany (Langgemach and Litzbarski 2005, Raab et al. 2010,

40   Langgemach 2012; http://www.grosstrappe.at; http://www.grosstrappe.de). Specifically for the

41   study population in the West-Pannonian region, conservation efforts since 2001 have centered on

42   implementing large-scale agri-environmental measures funded through Austrian and Hungarian

43   Agri-environmental Scheme for Great Bustard and Austrian, Hungarian and Slovakian Great

44   Bustard LIFE, LEADER, INTERREG and Rural Development projects to (1) improve the

45   quality of the habitat, (2) increase the species' reproductive success (details in Raab 2013;

46   http://www.grosstrappe.at, Faragó et al. 2014, Raab et al. 2014a, b), and (3) reduce power line

47   collision casualties, by burying powerlines or installing bird collision diverters (Raab et al.

48   2012). In spite of all these conservation efforts, the marked decreases in the West-Pannonian

49   population and in Germany in just a few decades might have caused already a loss of the original

50   genetic diversity and an increase in inbreeding in these populations.

51   Genetic analyses have been done in this species not only for its phylogenetic study (Pitra

52   et al. 2012, Arif et al. 2012, Horreo et al. 2014), but also for conservation purposes. These

53   studies have shown that Iberian and central European populations remain two distinct

54   evolutionary significant units (Pitra et al. 2000) despite their considerable dispersal and

55   migratory capacity. Moreover, significant genetic differentiation was suggested among local

56   populations within these two subpopulations, highlighting the need to conserve not only the

57   Iberian stronghold, but also all other smaller extant breeding groups of central Europe and Asia,

58   in order to preserve the current genetic pool of the species. An effective conservation program

59   needs a detailed knowledge of the genetic structure of the different subpopulations, but such

60   studies have only been published for the Iberian Peninsula and Morocco (Martin et al. 2001,

61   Broderick et al. 2003, Alonso et al. 2009, Horreo et al. 2014), with only limited data for other

62   European populations (Pitra et al. 1996, 2000).

63   The aim of this work is to explore for the first time the population structure, genetic

64   diversity, and gene flow in the Austrian-Slovakian-West Hungarian (known as West-Pannonian)

65   population of Great Bustards, using fourteen recently developed microsatellite loci (Horreo et al.

66   2013). The results should be useful not only for the conservation of this isolated central

67   European population, but also to preserve the genetic diversity of this globally endangered

68   species.

69

70   **MATERIALS AND METHODS**

71 During the 2012 and 2013 breeding seasons (between early April and early September), Great

72 Bustard moulted feathers were searched as DNA source in all areas previously known to be used

73 by the species in Austria. In Austria, the authors had verbal permission from hunters to collect

74 feathers in their properties. No other official permit is needed in this country to collect feathers.

75 As for Hungary, the authors had a permit to collect gene samples issued for the Hungarian LIFE

76 project (LIFE04 NAT/HU/000109), in which the Hungarian Ministry of Environment and Water

77 was a co-financier and the regional competent Fertő-Hanság National Park Directorate was a

78 partner. Single or small groups of feathers were found at 80 sites, covering the three most

79 important breeding areas in Austria: Weinviertel, Marchfeld and Heideboden (Figure 1). To

80 minimize the probability that two of the feathers collected for genetic analysis were of the same

81 individual we used only a single feather from each group of moulted feathers found at each of

82 these sites. Another 18 tissue and feather samples from the collections of the Vienna Natural

83 History Museum (years 2000-2006) were used, totalling 98 individuals. DNA was extracted from

84 the 80 feather samples and fourteen Great Bustard microsatellite loci were amplified three

85 independent times following protocols published in Horreo et al. (2014). Additionally, museum

86 DNA samples were checked for DNA quality before amplification and their PCRs were done in

87 laminar flow cabinets. These museum samples did not affect our analyses since the species'

88 longevity is estimated at 10-15 years (own unpublished data). Introgressive laser signals from

89 one channel to another during genotyping were carefully controlled. In order to avoid resampling

90 of individuals, the genetic Identity Analysis of the Cervus software v.3.0 (Kalinowski et al.

91 2007) was carried out.

92   Microchecker was employed to search for null alleles, scoring errors and large allele

93 dropout (VanOosterhout et al. 2004). Linkage disequilibrium was tested with Genepop on line

94   software (http://genepop.curtin.edu.au). Deviations from Hardy–Weinberg equilibrium after

95   Bonferroni correction (HWE), the number of alleles per locus (Na), the expected and observed

96   heterozygosities averaged across loci (respectively, H_S and H_O), the inbreeding coefficient (F_{IS})

97   and the Fixation Indexes (F_{ST}) and their associated P-values, were calculated with GenoDive

98   2.0b25 (Merimans and Van Tienderen 2004). Allele frequency distribution was plotted with

99   GenAlex v.6.5 (Peakall and Smouse 2012). Allelic Richness (AR) was estimated with FSTAT

100   v.2.9.3.2 (Goudet 1995). The presence of past genetic bottlenecks was tested with the software

101   Bottleneck v.1.2.02 (Piry et al. 1999), with the most suitable settings for microsatellite loci data:

102   the two-phase model (TPM) assumptions estimating P-values with the Wilcoxon signed-rank test

103   (10,000 interactions).

104   Several different approaches were used to ensure robust quantification of the population

105   differentiation in the study area. The Bayesian clustering method and Markov Chain Monte

106   Carlo (MCMC) simulation implemented in STRUCTURE 2.3.4 (Pritchard et al. 2000) were used

107   to assess population structure. The STRUCTURE analyses were run by using an admixture

108   model and correlated allele frequencies with a burn-in period of 50,000 replicates and sampling

109   period of 500,000 replicates for number of clusters (K) from 1 to 3. Ten independent runs were

110   performed for each K. We run two sets of STRUCTURE analyses: one without using sample

111   location as prior, one using sample location as prior (LocPrior option). To determine the number

112   of genetic clusters (K), we used the DK method of Evanno et al. (2005) based on the second

113   order rate of change in log Pr (X|K) as implemented by the program Structure Harvester v.0.6.94

114   (Earl and vonHoldt 2012). The second approach was BAPS v.6.0 (Corander et al. 2008), which

115   given a maximum value of partitions, uses a stochastic optimization procedure to find the

116   clustering solution with the highest marginal likelihood of K (i. e., an approximation of the most

117   probable number of differentiated genetic populations conditional on observed data). Settings:

118   admixture of individuals based on mixture clustering after clustering of groups of individuals;

119   1000 iterations used to estimate the admixture coefficients for the individuals; 200 reference

120   individuals from each population (as recommended by the software developers); 10 genetically

121   diverged maximum groups used to estimate the admixture coefficients for the reference

122   individuals. The third approach was a discriminant analysis of principal components (DAPC), a

123   multivariate method implemented in the *adegenet* package (v. 1.3-1) for the *R* software (Jombart

124   et al. 2008) that identifies clusters of individuals without using any population genetic model.

125   We used it in two different ways: (1) using the *find.clusters* function for the identification of the

126   optimal *K* with the *choose.n.clust* option and the Bayesian Information Criterion (BIC); after

127   that, DAPC was employed to assign individuals into populations, retaining the n/3 number of

128   principal components (as recommended in the manual) with 80% of the cumulative deviance

129   (which removes the effect of assigning populations a priori on the eventual assignment to

130   clusters and offers an unbiased interpretation of population structure); and (2) without searching

131   and optimal *K*: each individual was a priori assigned to its location of origin, obtaining for each

132   individual the probability of assignment to their populations of samplings. Lastly, Oncor

133   (Kalinowski et al. 2008) Leave-One-Out test assignments (default settings) were employed also

134   for searching/confirming population structure and for confirming Structure/BAPS/adegenet

135   results. These analyses delete each individual of the total dataset and try to assign it to one of the

136   given populations; results show percentages of correct assignations for each population. The

137   effective sizes (N_e) of the genetic estimated units were calculated with LDNe v.1.31 software

138   (Waples and Do 2008) employing random mating and 0.05 lowest allele frequency settings.

139   Contemporary gene flow among populations was studied under the assignment test

140   criterion of Rannala & Mountain (1997) with Geneclass 2.0 (Piry et al. 2004), which detect

141   individuals with immigration ancestry of up to two generations ago. The numbers of migrants

142   per generation (Nm) among breeding areas were estimated by the mean frequency of private

143   alleles with the above-mentioned Genepop software.

144

145   **RESULTS**

146   Two of the fourteen available microsatellite loci were discarded from analyses for different

147   reasons: the Ot3 locus did not amplify (probably due to mutation(s) in the primer(s) area(s)) and

148   the Ot6 locus was monomorphic in the dataset. Within the other twelve loci, amplification

149   success was 87.4% and Microchecker discarded the presence of scoring errors, large allele

150   dropout and null alleles in the dataset. The Identity Analysis showed two individuals with the

151   same genotype in the Weinviertel breeding area, one of which was deleted in order to avoid

152   resampling. The genetic variability (Table 1; Figure 2) was high: the mean number of alleles per

153   locus ranged between 3.25 (Marchfeld) and 5.17 (Heideboden), and the allelic richness ranged

154   between 3.07 (Marchfeld) and 3.60 (Heideboden). The smallest observed and expected

155   heterozygosities were found in Heideboden (0.42 and 0.49, respectively), and the highest in

156   Marchfeld (0.50 and 0.52, respectively). The inbreeding coefficients ranged between 0.03 in

157   Marchfeld and 0.13 in the Heideboden, being positive in all three breeding areas, and no genetic

158   bottlenecks were found in any of them. Non-significant deviations of HWE were found in 8, 9

159   and 5 out of 12 cases for Weinviertel, Marchfeld and Heideboden, respectively. Deviations from

160   HWE were caused by heterozygote deficit. The Ot12 microsatellite locus showed significant

161   HWE deviations in all the three breeding areas, so all analyses were done including and

162   excluding it, with identical results in both cases (only results with this microsatellite locus are

163   shown in the manuscript). Linkage disequilibrium was only statistically significant in a very

164   small proportion of tests (3.03%).

165   According to Structure Harvester, Structure results (with and without LocPrior option)

166   did not reveal a clear population structure across the study area, showing a unique genetic unit (K

167   = 1) in the whole dataset. Even forcing $K = 2$ or 3, results did not show differences among

168   breeding areas, they only showed mixed genotypes across all the three sampling points. Despite

169   this, BAPS analyses showed the presence of two genetic subunits ($K = 2$; Figure 3A) in the

170   dataset, one composed by Weinviertel and Marchfeld, and another including the Heideboden

171   individuals. DAPC analyses with the *find.clusters* function also revealed $K = 2$, but membership

172   results showed no correspondence between genetic and spatial structures/populations (Figure

173   3B), indicating the existence of a unique genetic unit (as STRUCTURE suggested). When each

174   individual was a priori assigned to its location of origin, obtaining for all individuals the

175   probability of assignment to the populations where they were sampled, more similar results to

176   the BAPS ones were found (Figure 3C). This could mean that $K = 2$ is a biased interpretation of

177   population structure. However, Oncor Leave-One-Out tests showed that when the three breeding

178   areas were employed as independent areas, percentages of correct individual assignments were

179   64.7, 71.4 and 85.7 % in Weinviertel, Marchfeld and Heideboden, respectively, whereas

180   employing two subunits (following BAPS results) yielded higher assignment values: 95.8% of

181   correct individual assignment for the genetic subunit composed by Weinviertel and Marchfeld,

182   and 85.7% of correct individual assignment for the other genetic subunit (Heideboden). In

183   addition to this, and in accordance with BAPS and Oncor results, F_{ST} P-values were not

184   significant between Weinviertel and Marchfeld and highly significant (P-val < 0.01) between

185   Heideboden and the other two breeding areas (Table 2). For all these reasons, the effective

186   population sizes (N_e) were estimated for the two mentioned genetic subunits, resulting in 30.3

187   individuals (18.4 - 58.1; 95% C.I.) for the subunit composed by Weinviertel and Marchfeld and

188   57.1 individuals (28.5 - 247.9; 95% C.I.) for the other subunit (Heideboden).

189   According to the assignment tests on recent gene flow, 66.3% of the individuals were

190   residents in the breeding area where they had been sampled when comparing the three sampling

191   sites, and 81.6% when comparing the two genetic subunits (Heideboden *versus* Weinviertel plus

192   Marchfeld). The number of migrants per generation (Nm; Table 2) was high in all cases (Nm >

193   2), being higher between Heideboden and the other two breeding areas (Weinviertel and

194   Marchfeld) than between the two latter. The Nm value between Heideboden and the genetic

195   subunit including Weinviertel and Marchfeld individuals was 3.62, the highest of all estimated

196   Nm values.

197

198   **DISCUSSION**

199   Our results show that the West-Pannonian population of Great Bustards displays high genetic

200   variability (Na = 3.25-5.17, AR = 3.07-3.60, He = 0.49-0.52) in spite of a strong decrease in

201   numbers of individuals suffered in the past decades, with a slightly smaller number of alleles per

202   locus but higher allelic richness than the world's largest populations of this species (Na = 4.5-5.9,

203   AR = 1.47-1.59, He = 0.50-0.59 without Ot3 and Ot6; Horreo et al. 2014 and unpublished own

204   data). This, together with the results showing absence of genetic bottlenecks in this area,

205   suggests that the marked demographic decline suffered by West-Pannonian Great Bustards

206   during the last century has not affected them in this respect, partly because the intensive

207   protection measures probably stopped early enough the population decline (Raab et al. 2010).

208   Overall, the genetic variability of the West-Pannonian population will probably be useful for

209   avoiding major genetic changes (as genetic drift) in the near future and thus for maintaining the

210   favourable trend of this population, provided that current habitat structure, climate conditions,

211   management practices and other factors affecting this population remain constant in the future.

212   The West-Pannonian population showed also to be genetically sub-structured despite the

213   relatively small area it occupies (Figure 1). All population structure analyses used revealed the

214   presence of a single population structure across the study area, with a hidden subpopulation

215   structure (as found in different geographic regions in other bustard species; Riou et al. 2012): the

216   birds in Heideboden (the largest, southern breeding group) were genetically different from the

217   birds in other groups, forming a genetic subunit on their own, while the other two breeding areas

218   (Weinviertel and Marchfeld) constitute together a second genetic subunit with a smaller effective

219   size. In addition to this, subdivision into units with distinct gene frequencies creates a

220   heterozygote deficit, so deviations from HWE in our dataset, caused precisely by heterozygote

221   deficit, reinforce the existence of this sub-population structure within the entire population.

222   The above-mentioned genetic differences cannot be attributed to physical barriers since

223   there are no such geographic obstacles between Heideboden and the other two breeding areas

224   (Figure 1). As in other bustard species (e. g. Idaghdour et al. 2004), contemporary gene flow (up

225   to two generations) was detected in the dataset, and Nm values were higher than one migrant per

226   generation (OMPG; Mills & Allendorf 1996) in all cases, including both gene flow among

227   breeding areas and between the two genetic subunits. The gene flow inferred from high Nm

228   values and low genetic differentiation can be the result of a relatively recent demographic

229   separation, in numbers of generations after the split. Despite this, the three breeding areas are

230   separated by distances that can easily be covered by dispersing juveniles (Martín et al. 2008),

231   and an exchange between the different Great Bustard breeding areas within the study area was

232   indeed suggested based on local field observations of bird movements (Raab 2013) as one of the

233   possible causes for the rapid recovery of this population (Raab et al. 2010). These gene flow

234   values (Nm = 2.09-3.27) were also in accordance with those found for the species in other areas

235   (Nm = 2.0-7.6 within genetic units in Spain; Horreo et al. 2014). Genetic differentiation and gene

236   flow due to recent demographic isolation/gene flow barriers could be therefore discarded. The

237   results on structure and gene flow patterns lead us to propose the whole area as a unique Great

238   Bustard population conformed by two genetic subunits.

239   In addition to this, the whole West Pannonian population is not far from other Hungarian

240   breeding groups, from which considering again the dispersal capacity of the species (Martín et al.

241   2008), dispersing immature individuals could have arrived and established at any of the breeding

242   groups in Austria as breeding adults, contributing to keep the genetic diversity high. In

243   particular, this could be the case in Heideboden, which is the West-Pannonian breeding

244   aggregation closest to the Hungarian groups. Our study showed that Heideboden is included

245   within the whole population, but genetically differs from the other two Austrian subunits, having

246   the highest genetic variability and effective size. This subunit, the largest among all breeding

247   groups in the study area, seems to be not only the connection among central Hungarian and

248   Austrian populations, but also a possible source of individuals for the other West-Pannonian

249   genetic subunit (Nm between Heideboden and the other Austrian subunit was higher than Nm

250   among breeding areas). These hypothesised movements could be confirmed through marking

251   individuals. Therefore, within the conservation and management plan for the future, all extant

252   breeding groups should be protected, but paying special attention to the Heideboden subunit,

253   since due to its proximity to central Hungarian populations it could be crucial. This subunit

254   should be therefore a priority for the conservation of other West-Pannonian Great Bustard

255   breeding groups, and to guarantee their connection to the main Hungarian populations further

256   east.

257   Summing up, our study is the first description of the genetic diversity and gene flow of

258   the West-Pannonian population of Great Bustards based on analysis of microsatellite loci. This is

259   one of the few populations that survived the sharp decreases that decimated this globally

260   endangered species during the last century in central Europe. Our results identified one

261   population in the study area, formed by two genetic subunits with high migration rates among

262   them. The current genetic variability is high. Conservation efforts should be directed not only to

263   preserve all extant breeding groups, but also the connectivity among them and with central

264   Hungarian populations, in order to keep the genetic diversity of West-Pannonian Great Bustards

265   at a high level. Current management and protection actions should therefore be maintained in the

266   study area in order to guarantee the survival of this Great Bustard population.

267

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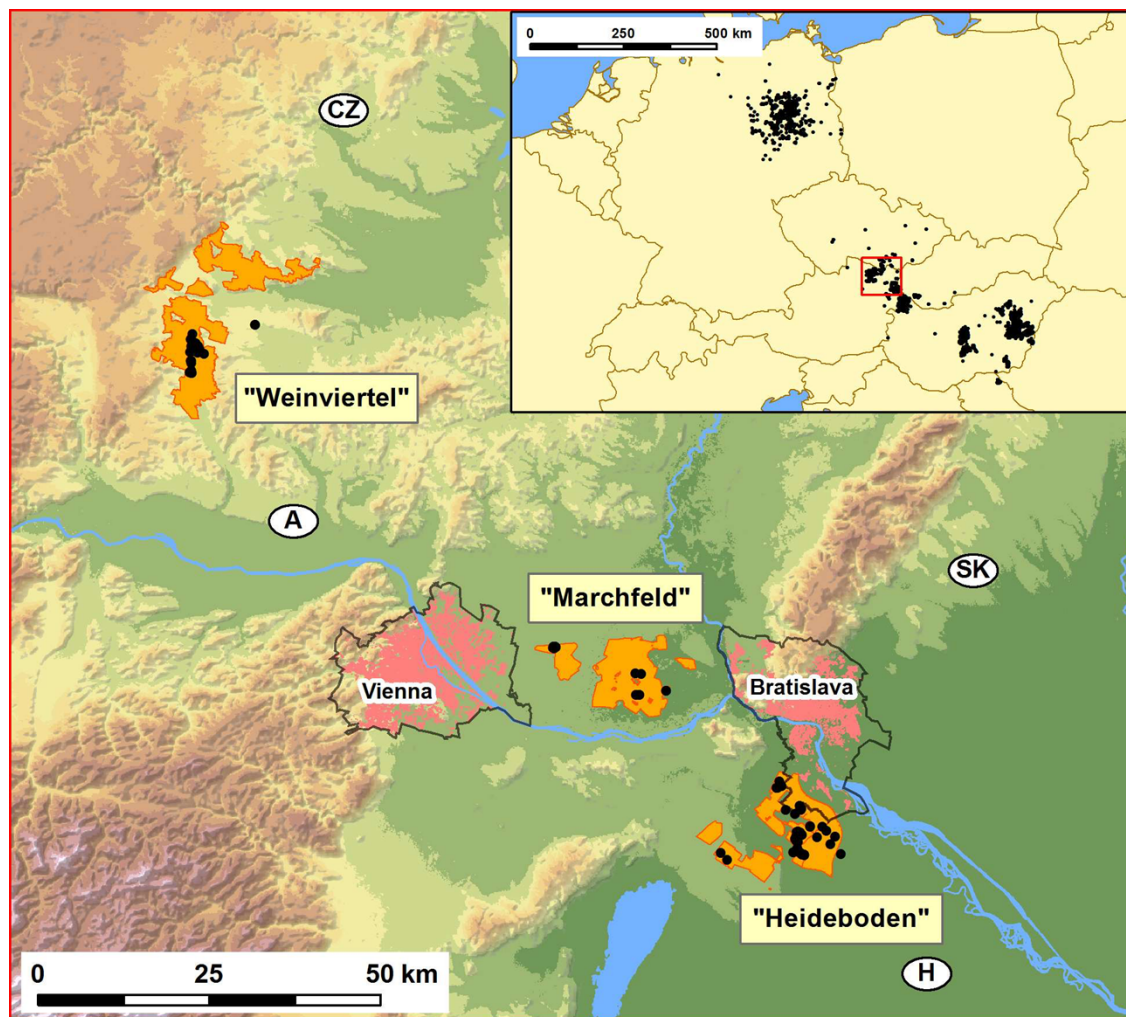
375

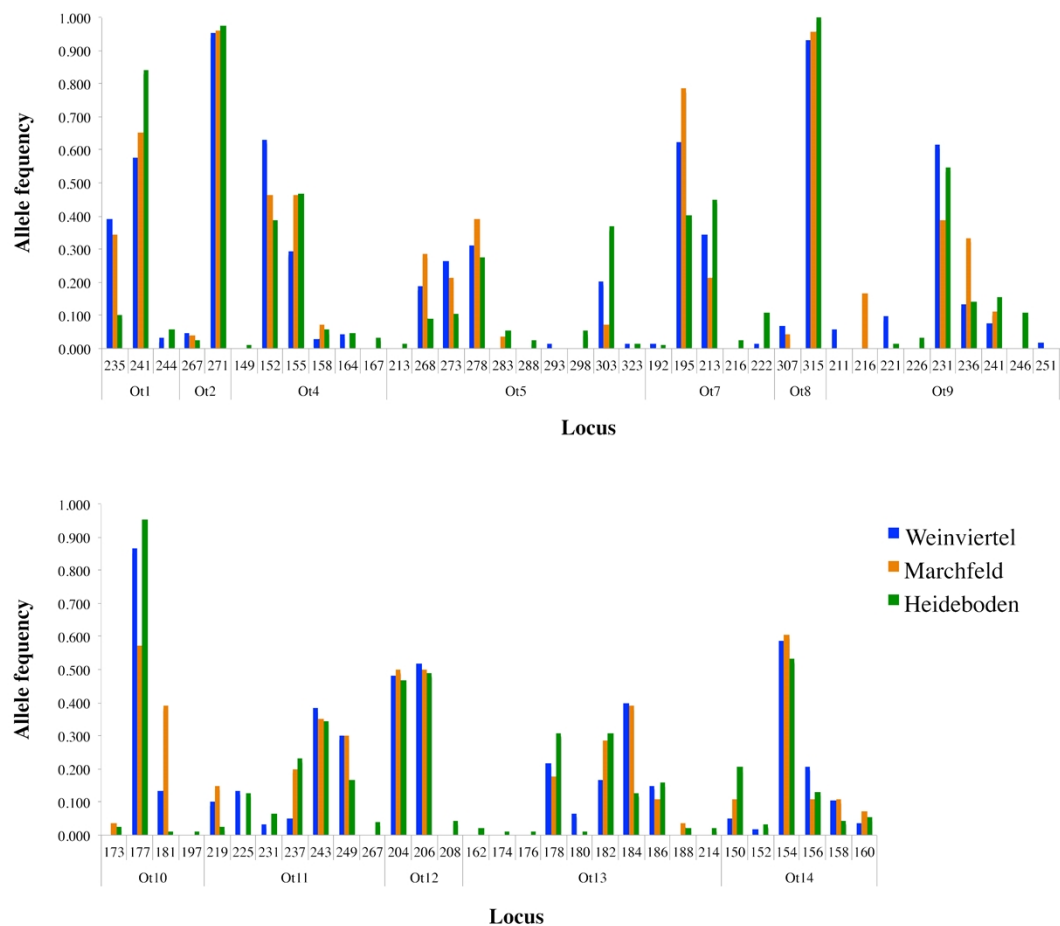
376

378 **FIGURES**

379 **Figure 1.**

380

381 **Figure 1.** Map showing the distribution of the studied Great Bustard breeding areas in Austria-
382 West Hungary-Slovakia (Weinviertel, Marchfeld and Heideboden; orange patches), and the
383 location of the study area within central Europe (inset map on the upper right corner). These
384 three orange areas include, respectively: SPA "Westliches Weinviertel" (AT1209000), ca.7441
385 ha; a large part of SPA "Sandboden und Praterterrasse" (AT1213V00), ca. 11.083 ha; and SPA
386 "Parndorfer Platte – Heideboden" (AT1125129) -ca. 7260 ha- plus the northern part of
387 Hungarian SPA "Mosni-sík" (HUFH10004) -ca. 3159 ha- and the Slovakian SPA "Sysl'ovské
388 polia" (SKCHVU029) -ca. 1777 ha-. The black dots indicate the collection sites of the samples
389 used for genetic analyses. The two capital cities, Vienna and Bratislava (pink patches), the
390 perimeters of the suburban areas around them (black lines around pink patches), and the initials
391 of the four countries converging in this geographic region (Austria, Czech Republic, Slovakia
392 and Hungary), and their national borders (black lines) are also indicated. The inset map shows
393 the distribution in central Europe in the period 1995-2014 (Source: data base from Rainer Raab,

394 including information from Péter Spakovszky and Miklós Lóránt for Hungary and Torsten
395 Langgemach and Henrik Watzke for Germany).

397 **Figure 2.**

398
399
400 **Figure 2.** Allele frequency distribution of the twelve analysed microsatellite loci across the three
401 breeding areas.
402

403

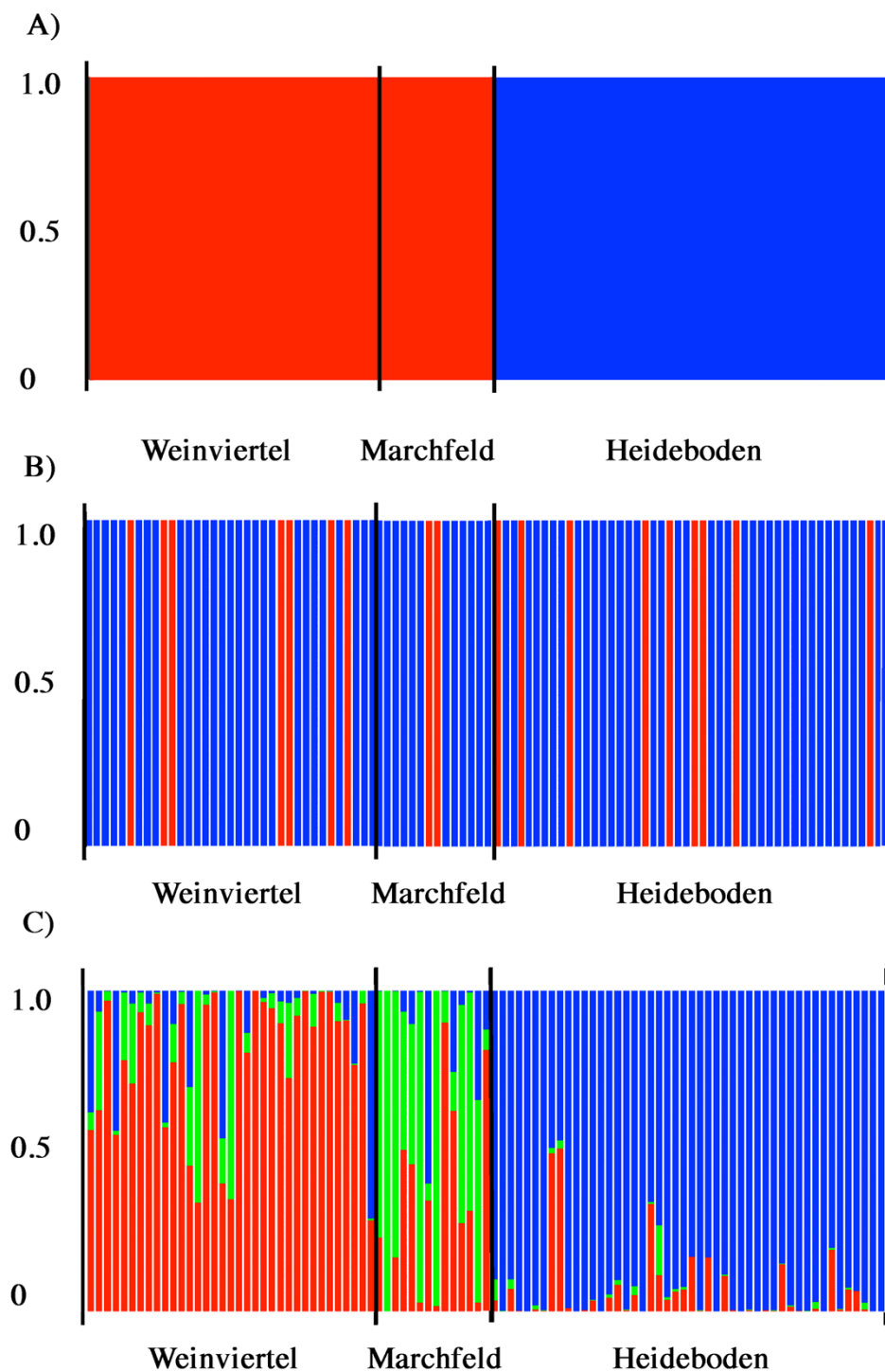



Figure 3. Output of BAPS (A) and DAPC analysis with (B) and without (C) the *find.clusters* function showing the membership fraction (columns) of the inferred genetic units for West-

406   Pannonian Great Bustards in each individual of the three studied breeding areas. Same colour in
407   different individuals indicates that they belong to the same cluster.

408 **TABLES**

409 **Table 1.-** Number of alleles per locus and outcome of tests for deviation from Hardy-Weinberg proportions (*$P<0.05$, and **$P<0.01$)

410 after Bonferroni correction in the three sampled West-Pannonian Great Bustard breeding areas (see Fig. 1) for the 12 studied

411 microsatellite loci. Acronyms: Na, mean allele number per locus; AR, allelic richness; H_O, observed heterozygosity; H_E, expected

412 heterozygosity; F_{IS}, inbreeding coefficient; B, *P*-value for Bottleneck analyses. Standard deviation is shown between brackets.

413

	Ot1	Ot2	Ot4	Ot5	Ot7	Ot8	Ot9	Ot10	Ot11	Ot12	Ot13	Ot14	Na	AR	H_O	H_E	F_{IS}	B
Weinviertel n=35	3**	2	4	6	4**	2	6	2	6**	2**	5	6	4.00 (1.76)	3.27 (1.37)	0.44 (0.22)	0.49 (0.23)	0.11	0.67
Marchfeld n=14	2	2	3	5	2	2**	4*	3	4	2**	5	5	3.25 (1.29)	3.07 (1.22)	0.50 (0.28)	0.52 (0.23)	0.03	0.21
Heideboden n=48	3**	2	6	9	5**	1	6**	4**	7**	3**	10	6	5.17 (2.72)	3.60 (1.72)	0.42 (0.29)	0.49 (0.29)	0.13	0.27
415 **Table 2.-** Genetic differentiation among West-Pannonian bustard breeding areas pairs measured

416 with F_{ST} values (below diagonal; **P-value<0.01) and gene flow among them measured as the

417 number of migrants per generation (Nm; above diagonal).

418

F_{ST}/Nm	Weinviertel	Marchfeld	Heideboden
Weinviertel	-	2.39	3.07
Marchfeld	0.014	-	2.59
Heideboden	0.036**	0.055**	-

419