DIPLOMA THESIS

CORRELATIONS BETWEEN CHORUS SIZE AND GENETIC DIVERSITY IN *HYLA ARBOREA* IN THE REUSS VALLEY, SWITZERLAND

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Correlations between chorus size and genetic diversity in *Hyla arborea* in the Reuss valley, Switzerland

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Abstract

I investigated the relationship between genetic diversity and chorus size estimates in the European treefrog (*Hyla arborea)*. The European Tree frog is one of the most endangered amphibian species in Switzerland and only occurs in a few geographically distinct regions. For the metapopulation in the Reuss valley, comprehensive genetic and male chorus size data exists. For genetic parameters, I used the allelic richness, the number of alleles, the expected and the observed heterozygosity and the inbreeding coefficient of each of the 33 known subpopulations. For chorus parameters, I used the chorus size, the subpopulation trend and the coefficient of variation of the chorus size. Chorus size parameters were computed for the individual subpopulations and for genetically differentiated clusters of subpopulations. Furthermore I calculated the connectivity for each subpopulation. I then built models for the genetic parameters described by chorus count parameters and connectivity conducting model selection and model averaging. It appears that different genetic parameters are correlated to different parameters used in this study. Strong correlation was found for allelic richness and geographical connectivity. The number of alleles was related to male chorus size and the proportional variation in chorus size. The expected heterozygosity seems to be influenced solely by the proportional variation in chorus size. For the inbreeding coefficient, I found a correlation with the proportional variation in chorus size, but theoretical support for the resulting model is lacking and it even appears that the model does not make sense. The results of the present study are generally congruent with expectations based on theory. There exist biological explanations for most of my findings. Based on the results I recommend not only to use the population size but also the variation of the population size over time when looking for the influence on genetic parameters or when assessing the condition of populations.

Keywords: *Hyla arborea*, European Tree frog, microsatellites, genetic diversity, chorus size, connectivity, Reuss valley

Introduction

Amphibians show a worldwide decline (Houlahan *et al.* 2000) and currently one third of them are listed as threatened (IUCN 2007), more than in any other vertebrate taxon. Major causes for this global decline may be habitat destruction and degradation (Semlitsch 2000). The loss of habitats may lead to the extinction of populations. In addition, the reduction in quality of the remaining habitats may affect their size and condition. The surviving populations further suffer from impeded migration due to larger distances to other remaining populations and to anthropogenic barriers fragmenting the landscape. The combination of decreased size and increased isolation can lead to lower genetic diversity of the populations (Frankham 1996) and to consequential fitness handicaps (Reed & Frankham 2003, Edenhamn *et al.* 2000, Weyrauch & Grubb 2006).

All factors named above can certainly affect organisms other than amphibians. Nevertheless, amphibians are thought to be indicator species providing an early warning of natural responses to environmental impacts as they are dispersal-limited and rely on specific and complex habitat structure. Therefore their decline could serve as a model for understanding the global biodiversity crisis in general (Storfer 2003, Carignan & Villard 2001).

One amphibian species suffering heavily from habitat loss and fragmentation is the European Tree frog (*Hyla arborea)*. It is a demanding species, which needs temporary ponds (or permanent ponds without fish; van Buskirk 2003) that are preferably rich in plants but exposed to direct sunlight (Pellet *et al.*

2004*b*) for breeding. In addition, it requires nearby shrubberies or woods as terrestrial summer habitat. *H. arborea* never reproduces in forest ponds (Grosse 1994) and urbanization as well as traffic have a negative effect on its presence (Pellet *et al.* 2004*a* , Pellet *et al.* 2004*b*). Particularly due to these quality requirements on the landscape, *H. arborea* underwent a 50 percent range reduction in Switzerland during the 1980es (Grossenbacher 1988) and is now considered as endangered (IUCN 2007). Once widely spread in the Swiss lowlands, *H. arborea* today occurs only in few metapopulations each of them embedded within discrete regions (Zumbach 2004).

One such region is the Reuss valley (Figure 1), situated in the centre of the Swiss lowlands. In this densely populated and intensively farmed region the influence of the river is restricted to a small part of the valley floor. The region spans across the two cantons Aargau and Zürich and has been listed in the Federal Inventory of Landscapes and Natural Monuments since 1977 (Der Schweizerische Bundesrat 1977). Furthermore, the Aargau sector is protected by environmental legislation of the canton since 1983 (Regierungsrat des Kantons Aargau 1983). In both cantons, several inventories of *H. arborea* were carried out in the last decades. In 1991/92 the canton Aargau reported an overall decline of about 37 percent and the extinction of two of the last three metapopulations present in 1978/79. In canton Zürich, the inventory of 2002 (the third after 1967-69 and 1977-81) assessed for the first time a slight increase in the subpopulations in the Reuss valley but still alerted that the population's recovery is not guaranteed (Cigler *et al.* 2002).

The results of the inventories gave rise to protection efforts in both cantons. Pro Natura Aargau started a conservation project in 1991 including the protection of areas, the acquisition of land and comprehensive landscaping. By now almost all breeding sites are protected and in good condition. The canton Zürich enhanced several sites since 1995 (Fachstelle Naturschutz 2001) and maintains an action plan since 2004 (Fachstelle Naturschutz 2004). Mainly these efforts preserved *H. arborea* in the Reuss valley and prevented a further decline (Flory 2004).

To record the effect of these efforts, the metapopulation of *H. arborea* in the Reuss valley has been annually monitored since 1994. Volunteers check all sites one to three times during each breeding season and thereby estimate the number of calling males. Chorus count data serve as an index for subpopulation size and are often chosen in monitoring programs because of the comparatively small expenditure of time and money. Nevertheless, a problem arises because males are not calling during the whole breeding season (e.g. 17.0±10.1 nights, Grafe & Meuche 2005; 11.67±5.17 nights, Pellet *et al.* 2007*b*). It is therefore widely agreed that chorus size counts underestimate true male population size (Pellet *et al.* 2007*b*, Pellet & Schmidt 2005, Grafe & Meuche 2005). To assess this discrepancy I used a recent computer program, which estimates effective population size basing on Bayesian computations.

The usage of annual monitoring data to determine the success of the protection efforts is only sensible if chorus counts are correlated to genetic parameters. Such relationships are expected by theory (Frankham 1996), but empirical support for anurans is controversial. Allentoft *et al.* (in press) did not find significant correlations between expected heterozygosity and population size and the degree of isolation in *Bufo calamita*. Edenhamn *et al.* (2000) did not locate differences in genetic variation in relation to isolation in *H. arborea*. However, more knowledge of the situation of *H. arborea* is cruical because the genetic diversity is thought to affect the individual fitness and the adaptive potential. Less fit and less adaptive populations may suffer more from selection and changing environmental conditions. This effect could in turn influence the genetic diversity and therefore lead to a vicious circle decreasing the survival potential of the species (Willi *et al.* 2006). Hence, investigation of correlations between genetic and chorus data in an independent system like the Reuss valley (whose metapopulation of *H. arborea* is thought to be isolated) could be enlightening. Particularly, this knowledge could help optimize further conservation directions.

I therefore combined the monitoring data from 1999 to 2006 with a genetic data set by Sonia Angelone (WSL Birmensdorf, unpublished data) who sampled the entire metapopulation during the breeding season 2006. Thereby buccal swabs from 581 individuals were collected and processed with seven microsatellites. I expected to find the following patterns according to theoretical approaches. First, larger subpopulations should show greater genetic variation at neutral markers due to the assumption that more individuals contain more genetic information (Frankham 1996). Second, inbreeding should be lower in larger subpopulations as there are more potential mates enhancing random-mating. (Allendorf & Luikart 2007) Third, more isolated subpopulations located at the border of the area should exhibit lower genetic diversity and higher inbreeding due to less immigration. Besides, using only the genetic data, I checked each subpopulation of the Reuss valley for recent (0.2-4.0 *Ne* generations) bottlenecks, since the whole meatapopulation is suspected to have suffered from at least one drastic reduction of size during the last century. Genetic evidence for recent bottlenecks would highlight the necessity for protection and the absence of such an incident could deliver an indication for successful conservation efforts.

Materials and Methods

Chorus count data

The chorus size data of the *33* known subpopulations used in this study are provided by two different institutions, namely the "Amphibien-Monitoring Aargau" (Meier & Schelbert 1999) and the "Fachstelle Naturschutz Zürich" (Hunziker 2007). For the investigations, I used the maximum chorus counts per subpopulation and per year (i.e. Schmidt & Pellet 2005, Allentoft *et al.* 2008) during the peak of the breeding season which I defined as the time span between April 15th and June 15th. To characterize the subpopulations and their dynamics, I calculated the following three parameters for each subpopulation over the time period 1999 to 2006: the arithmetic mean of the maximum counts (*Ns*), the development tendency in the form of the slope of the regression line (*Ts*) and the coefficient of variation of the chorus size (*CVs*). Furthermore, I estimated the connectivity (*K*) of each subpopulation using the equation

$$K = \sum_{i \neq j} e^{-\alpha * d(ij)} * N_j$$

(Hanski 1999), where *d(ij)* is the distance between the subpopulations *i* and *j*, N_j is the size (my *Ns*) of subpopulation *j* and $1/\alpha$ is the mean dispersal distance of the considered species, which I set as one because of absence of reliable data for *H. arborea* (Pellet *et al.* 2007*a*). Finally, I used the six geographically defined genetic clusters inferred using STRUCTURE (Pritchard *et al.* 2000) from the study of Sonia Angelone (WSL Birmensdorf, unpublished data) and gave them following color names: pink, red, blue, green yellow and purple (Figure 1). I assigned the maximum chorus counts per year to each cluster and calculated the following parameters per cluster: the arithmetic mean of the maximum chorus counts (*Nc*), the subpopulation trend (*Tc*), and the coefficient of variation of the chorus size (*CVc*).

To test for the reliability of chorus size as an index of population size, I compared the chorus size with an estimated effective population size (N_e^) provided by the web based program ONeSAMP (Tallmon *et al.* 2008), which uses summary statistics and approximate Bayesian computations. ONeSAMP assumes all loci to be unlinked and neutral what is achieved by the data. As recommended by the authors, I changed priors on N_e^ to get an idea of the robustness of the estimation. I received estimations for all subpopulations with a genetic sample size greater than one. An estimation of the effective population size with the computer program LDNe (Waples & Do in press), which uses linkage disequilibrium data, was not possible in this study case since the lowest allele frequency used would have been too high to provide reliable results because the populations are small.

Genetic data

One part of the genetic data used in this study was produced by Sonia Angelone (WSL Birmensdorf, unpublished data), who extracted and eluted DNA following the protocol of Broquet *et al.* (2007) and processed it using seven microsatellite loci described by Arens *et al.* (2000; WHA1–9, WHA1–20, WHA1–25, WHA1–67, WHA1–103, WHA1–104 and WHA1–140). To enhance the quality of multilocus genotypes, I expanded the data set by processing four additional microsatellite loci described in Berset-Brändli *et al.* (in press); HA–A127, HA–B5R3, HA–D115 and HA–E2). The PCR was carried out in 7 µL multiplex reaction mixtures containing 3µL of template DNA, 1x QIAGEN Multiplex PCR Master Mix and 0.4 to 1 µM of each of the fluorescently labeled forward and reverse primers on a PTC100 Thermocycler (MJ Research). PCR amplification conditions for the additional primers were: 15 min at 95 °C, followed by 30 cycles of denaturing for 30 s at 95 °C, annealing for 90 s at 58 °C and extension for 90 s at 72 °C, and process closing with 10 min at 72 °C. Capillary electrophoresis was carried out on an ABI Prism 3130 Genetic Analyzer using GeneScan™-500 ROX™ Size Standard and the resultant peaks were analyzed in Genemapper 3.7 (all by Applied Biosystems).

I tested all loci for accordance with Hardy-Weinberg proportions using Fisher's exact tests as implemented in Genepop v.1.2 (Raymond & Rousset 1995). To check for non-random association between alleles at two loci I used Fstat v.2.932 (Goudet 2001), which includes a log-likelihood ratio G-statistic. Resulting values were adjusted using the sequential Bonferroni correction (Rice 1989) wherever the analysis included multiple comparisons.

To assess the genetic condition of the subpopulations, I used two kinds of genetic population parameters. First, parameters describing genetic variability, namely the allelic richness (*Ra*; computed in Fstat v.2.9.3.2 (Goudet 2001)), the observed number of alleles (*Na*), the expected (*He*) and the observed heterozygosity (*Ho*; all computed in Genetix v.4.05 (Belkhir *et al.* 1996-2004)). Second, a parameter describing heterozygote deficit or excess within populations, namely the inbreeding coefficient F_{IS} (computed in Fstat v.2.9.3.2 (Goudet 2001)). With the few exceptions listed below, the genetic population parameters were successfully calculated for all *33* subpopulations. For *Ra* I received results for 22 subpopulations since it is sensibly computed for sample sizes of more than 10 individuals, although it is generally insensitive to sample size. Therefore, I also assessed *Na* even though its comparison among subpopulations is limited for uneven sample sizes. *He* (Nei 1987) and *Ho* are generally insensitive to sample

size and even a few individuals tested are sufficient if the number of loci is large (Gorman & Renzi 1979). For F_{IS}, I obtained results for 30 subpopulations with sample sizes larger than one.

A summary of (some of) the genetic parameters (*Ra*, *Na*, *He*, *Ho* and F_{IS}), by the use of a principal component analysis (PCA), while keeping as much information as possible, was not feasible in this study.

To detect drastic recent reductions in population size, I carried out corresponding tests using the program BOTTLENECK v.1.2.02 (Cornuet & Luikart 1996). The evaluation embedded in BOTTLENECK is based on the assumption that recently (meaning within the last 0.2-4.0 Ne generations) reduced populations should exhibit an excess of heterozygosity under the assumption of mutation-drift equilibrium. To test the accordance between the observed heterozygosity and the heterozygosity expected from the observed number of alleles, I used a one-tailed Wilcoxon sign rank test, which is recommended by Piry *et al.* (1999) for less than 20 polymorphic loci. I assessed the Two-Phased Model (TPM) of mutation because it is thought to fit best for microsatellite loci (Di Rienzo *et al.* 1994). I followed the recommendations of Piry *et al.* (1999) for the calculations, such as a proportion of 95% Stepwise Mutation Model (SMM) and 5% of multi-step mutations, a variance among multiple steps of 12 and a replication number of 1000.

Relationships

The aim of my study was to look for correlations between the genetic parameters (*Ra*, *Na*, *He*, *Ho*, F_{IS}) and the chorus count parameters (*N*, *T*, *CV*) of the subpopulations and the clusters plus the connectivity of the subpopulations (*K*). Data analysis was carried out using R v.2.6.1 (R Development Core Team 2007).

I started with a two-sided Kolmogorov-Smirnov test to check for normal distribution of the residuals. If necessary, I transformed the genetic parameters using a Box-Cox transformation (Box & Cox 1964). Because the independent variables *N*, *T*, *CV* and *K* exhibited a great range of variance, I scaled them to ensure a reliable assessment of their influence on the genetic parameters.

I tested the genetic parameters against the chorus count parameters and the connectivity as well as their combinations. Therefore I conducted a two-step analysis including a model selection followed by a model averaging. I carried out the model selection within the four groups of independent parameters *N*, *T*, *CV* and *K* and used linear and quadratic functions to describe the relationships (see also Table 1). Using Akaike Information Criterion

$$AIC_i = -2*\log L + 2*edf$$

and Akaike Weight

$$(AICw_i = e^{(-0.5*\Delta AICi)} / (\sum e^{(-0.5*\Delta AIC)}$$

(Burnham & Anderson 1998), I selected all models with an $AICw_i$ greater than 0.05. The selected models were brought together and treated as follows: If all selected models were composed of parameters of the same group, I built the final model using all components of the selected models. Otherwise, I carried out a model selection. I therefore combined the models with unequal components among each other and calculated these composed models. Finally, I averaged the composed models using the formula for the weighted model averaged parameter

$$\bar{\Theta} = \sum \pi_i * \Theta_i$$

(Burnham & Anderson 1998), where Θ_i is the value of the parameter and π_i is the probability of selecting each model, here $AICw_i$. In order to assess the importance of each parameter in the final model, I computed a 95% confidence interval using the averaged standard error, derived from the averaged variance

$$var(\bar{\Theta}) = \{\sum \pi_i * \sqrt{[var(\Theta_i \mid M_i) + (\Theta_i - \bar{\Theta})]}\}^2$$

(Burnham & Anderson 1998). To interpret the models, I plotted the genetic parameters against the chorus count parameters.

Results

Chorus count data

The arithmetic means of the maximum chorus counts, the subpopulation trends and the coefficients of variation of the chorus size for each subpopulation (*Ns*, *Ts* and *CVs*) as well as for each cluster (*Nc*, *Tc*, *CVc*) are given in Table 2. Note that a negative subpopulation trend is only exhibited in the pink cluster that consists of a single subpopulation, whereas clusters with more than one subpopulation seem to provide a rather constant environment. First, this fact is suggested by the pattern of negative population trends. The purple cluster, containing eleven subpopulations, includes four subpopulations with negative population trends. Overall, however but the cluster develops slightly positively. Second, the *CVc* of the purple cluster, which contains the four subpopulations with the by far highest *CVs*, lies in the range of other clusters.

Connectivity values (*K*) ranged from 0.2 to 170.7 and it is noteworthy that the values were lowest for the subpopulations lying within the pink and purple clusters, which are both isolated from the other clusters. However, the purple cluster exhibits a much higher *K* than the pink because it comprises eleven subpopulations while the pink consists of only one.

The comparison of the genetic sample size and the

median of the estimated effective population size (N_e^; by ONeSAMP) shows an astonishing high correspondence ($R^2 = 0.89$; trend line: N_e^ = 1.0422 * n + 0.5132). This result is quite robust since different priors on N_e^ (1-1000 and 4-500) produced similar results. I tried to check dependency on sample size by leaving out some individuals of a large population for test-calculations. But the web-based program did not accept any file after a certain point in time. Further, the authors did not have an explanation for the problem. For all these reasons I do not recommend to use the estimated N_e in this study to assess the quality of the chorus size or to calculate the time span where a possible bottleneck occurred. I rather suspect that ONeSAMP is not calculating properly.

Genetic data

Deviations from Hardy-Weinberg proportions after applying sequential Bonferroni corrections occurred only at a single locus (WHA–140) and a single subpopulation (GME) due to heterozygote excess. There was no evidence for significant non-random association between alleles at two loci after applying sequential Bonferroni correction. I found high levels of genetic diversity as the number of alleles per locus varied between two and 17 and I detected 12 private alleles out of a total of 100 alleles (five in AU and one in each of the subpopulations GSP, WIL, KLO, SCW, SLG, LOR and GRI). One locus was monomorphic in all populations of the yellow cluster and six loci were monomorphic in four populations of the purple cluster. The parameters describing genetic variability, as well as the inbreeding coefficient (F_{IS}) for each subpopulation across all loci are given in Table 2. Overall F_{IS} was slightly negative, but the surplus of heterozygotes compared to expectations under Hardy-Weinberg proportions was not significant (F_{IS} = -0.007, 95% CI: -0.03 to 0.017).
BOTTLENECK reported significant heterozygosity excess in three subpopulations, two of which are located in the purple cluster (GME, p = 0.00049; SLG, p = 0.00122) and one in the green cluster (FOO, p = 0.0105). Since the computed effective population sizes are affected by a high level of uncertainty, I did not determine the time period of a detected or a non-detected bottleneck, respectively.

Relationships

Box-Cox transformation was necessary for the expected (*He)* and the observed (*Ho*) heterozygosity and the inbreeding coefficient (F_{IS}) and resulted in log-transformed parameters: $tHe = \ln(He^4)$, $tHo = \ln(Ho^4)$, $tF_{IS} = \ln((F_{IS}+1)^2)$.
The model selection indicated that *Ho* is influenced by many factors (eight showed an $AICw_i > 0.05$; Table A, Appendix). I omitted building a final model for *Ho*, because it would have been very complex, inhibited detailed interpretations and exhibited a total AICw of only about 70%. For *Ra*, *tHe* and tF_{IS}, I built the models in the model selection (Table A, Appendix) whereas *Na* required model averaging (Table B, Appendix). Different genetic parameters were best explained by different chorus factors; no single chorus factor best explained all genetic parameters (Table 3). Not all chorus parameters are important, nowhere did *T* reach an $AICw_i$ larger than 0.05 and thus does not appear in any model.
The resulting models are reliable. Confidence intervals of the factor slopes overlap zero for six variables in two models (Table 3). In addition the AICw of the models are high except for tF_{IS} (Table A, Appendix). *Ra* exhibits a clear increase with increasing connectivity of the subpopulations whereby the effect shrinks for increasing *K*. At the upper end of the scale *Ra* even decreases (Table 3, Figure 2. a). *Na* is positively influenced by the chorus size and negatively influenced by the coefficient of variation of the chorus size. The cluster affects *Na* in the way that a large chorus size as well as a large *CVc* can increase *Na* of a subpopulation with distinct size or distinct *CVs*, respectively, whereas small chorus size and small *CVc* can decrease it (Table3, Figure 2. b & c). *He* exhibits an almost even correlation with *CVs* as long as *CVs* values are smaller than one. Beyond one (i.e. standard deviation exceeds mean number of calling males), expected heterozygosity decreases rapidly. Furthermore, a cluster with a smaller *CVc* can positively influence *He* of a subpopulation with distinct size, whereas a larger *CVc* can influence it negatively (Table 3, Figure 2. d). Finally, the model for F_{IS} shows a rigorous decline for high values of *CVs* and a humped correlation for smaller values. A smaller *CVc* seems to positively affect F_{IS} of a subpopulation with distinct *CVs*, while large *CVc* seems to affect it negatively (Table3, Figure 2. e).

Discussion

Correlations

I found correlations between genetic parameters and chorus count parameters as well as between genetic parameters and connectivity. Thereby, different genetic parameters are correlated to different parameters used in this study. The results of the present study are generally congruent with expectations based on theory. There is no evidence for contradicting findings. Nevertheless, not all models built in this study are explicitly interpretable. Even, some seem to make no sense. Since I used linear and quadratic functions to describe the models, the

accuracy is not as high as if I had included more complicated functions.

The correlation of the allelic richness (Figure 2. a) to the connectivity is widely supported by theoretical considerations. The connectivity of a subpopulation combines the distances to the other subpopulations and their size. For a higher connectivity I can therefore assume more migrating individuals and due to the larger genepool an increase of the genetic diversity, subsequently (Willi *et al.* 2006). The increase of allelic richness shrinks for increasing connectivity and approaches an asymptote because the number of individuals in the metapopulation is restricted. Above a certain point, a further increase of *K* does not create more varied genetic information. The decline of allelic richness at high connectivity values may be a simple consequence of the quadratic function used. Empirical support for the importance of connectivity for *H. arborea* is delivered by Vos and Stumpel (1995). They found that ponds occupied by *H. arborea* are less isolated than unoccupied ones. Other studies investigating the influence of isolation on genetic diversity did not find significant impact (Edenhamn *et al.* 2000, Allentoft *et al.* 2008). Also a more general study on ten species could not improve the model-predictions of colonization patterns by the inclusion of connectivity data Pellet *et al.* (2007*a*).

Direct correlation between the number of alleles and the chorus size found in the present study confirms expectations of theory whereupon larger subpopulations should show greater genetic variation (Willi *et al*. 2006). Furthermore, a large cluster may provide a larger allele-pool than a small cluster and is therefore supposed to lead to a higher number of alleles of subpopulations with distinct sizes. The number of alleles is thought to be most sensitive to the loss of genetic variation because of small population size (Allendorf & Luikart 2007). It is therefore not astonishing that for this parameter only I found a model including the population size. Besides small population size, also large relative variation of the subpopulation size over time reduces the number of alleles. This result is possible since each population size reduction decreases the number of alleles. However, sensible biological explanation for the model relating the number of alleles to the proportional variation of the cluster size is absent. Additionally, the shape of the curve is not comprehensible. However, the results should be treated with caution since the number of alleles depends on genetic sample size (Allendorf & Luikart 2007).

Only large relative variations in subpopulation size seem to affect expected heterozygosity (Figure 2. d). As long as the standard deviation does not exceed the subpopulation size, the expected heterozygosity is independent of chorus size. But above the threshold of $CVs = 1$, expected heterozygosity declines rapidly. The robustness of the expected heterozygosity for small and intermediate values of relative variation of chorus size could be expected. Allendorf and Luikart (2007) attribute a certain robustness to the expected heterozygosity regarding the influence of small population size (and therefore also to the variation of the population size as long as the population trend is not strongly increasing). Also the qualitative trait of the model relating the expected heterozygosity to the relative variance of the cluster is congruent with biological expectations. A larger relative variance of the cluster is supposed to lead to lower values in expected heterozygosity because it indicates fluctuations (unless the population trend is highly positive). However, the reasons for the shape of the model-curve remain unclear. Empirical results of studies investigating the influence on the expected heterozygosity are inconsistent. Andersen *et al.* (2004) investigated *H. arborea* in 12 ponds in Denmark and found significant correlation between the number of calling males and the expected heterozygosity based on 12 microsatellite loci. On the other hand, Allentoft *et al.* (in press) found no positive correlation between expected heterozygosity and population size for *Bufo calamita* in Denmark. Also the allelic richness was not significantly correlated.

In contrast to my assumptions neither the connectivity nor the subpopulation size seems to have an influence on the inbreeding coefficient. Nevertheless, such relationships cannot be excluded. The model describing the inbreeding coefficient is not supported by theory. Furthermore, the shape of the model-curve seems to make no sense.

Due to the findings that correlations of the number of alleles and the expected heterozygosity are influenced by the relative variance in chorus size, I suspect a considerable importance of (repeated) fluctuations in subpopulations. Even a later counterbalance of the subpopulation size seems to be unable to compensate for the loss of genetic diversity. This outcome is known as the “bottleneck” effect (Allendorf & Luikart 2007). However, support by empirical studies for my findings is rather poor. I found no study correlating variability of the population size to genetic parameters but some correlating the population/chorus size to genetic diversity.

Because genetics seem to be influenced by chorus size, as theory predicts (Willi *et al.* 2006) and several studies reported for amphibians (e.g. Edenhamn *et al*. 2000; Weyrauch & Grubb 2006) , there exists a need for deeper knowledge of the importance of genetics. Potential response to selection and individual fitness may be only two fields for further investigations. Additionally, my results could maybe give rise to a closer look on the effect of repeated reductions in population size. Future studies could possibly include

the variability of population size investigating correlations between population size and genetics.

Chorus count data

Clusters containing more than one subpopulation seem to be rather robust to negative subpopulation trends and high *CVs* values of individual subpopulations within clusters. No cluster shows negative a trend even if some of its subpopulations do. Effects from breeding site degradation can widely be excluded since the sites of both clusters are protected and managed for amphibians. Further, as both clusters are isolated from others, migration to and from other clusters is rather unlikely. In this sense the development in AU could be a cause for concern and needs to be monitored closely in the coming years. Second, high coefficients of variation of the subpopulation size appear not to have a stringent influence on the cluster, which could be an indication on migration between subpopulations of a cluster. The high *CV*-value of the yellow cluster has to be put into perspective. There is an impressive chorus size increase in all (!) of its subpopulations from 2005 to 2006 (2005/06: Nc = 61/111, N(DIC) = 21/35, N(TOR) = 5/20; N(SCW) = 34/52; N(EIC) = 1/3). Additionally, the yellow cluster consists of only five subpopulations, some of which show missing chorus size estimates for some years. Therefore, either a buffering effect or migration within the cluster could have been missed.

Genetic data

Two existing microsatellite studies of *H. arborea* show smaller ranges of expected heterozygosity than I found (0.27-0.68). Andersen *et al.* (2004) documented a range from 0.35 to 0.53 in Denmark, Arens *et al.* (2006) a range from 0.39 to 0.58 in the Netherlands. Also in other anurans smaller ranges were documented (0.38-0.71 in *Alytes mutensis* in Spain (Kraaijeveld-Smit *et al.* 2005); 0.18-0.43 in *Bufo calamita* in Denmark (Allentoft *et al.* in press) and 0.242-0.376 in Britain (Rowe *et al.* 2000); 0.43-0.8 in *Litoria aurea* in Australia (Burns *et al.* 2004)). Willi *et al.* (2006) expect a larger variability in genetic variance in small population sizes, because the response to selection is suspected to be more variable. Though the smallest subpopulations are found in the present study, neither the median nor the arithmetic mean of the population sizes of the mentioned studies support this prediction.

Bottlenecks

Three populations in two different clusters exhibited a significant heterozygosity excess. While it may indeed denote bottlenecks in the two subpopulations in the purple cluster, the subpopulation in the green cluster may have experienced a founder effect. At this latter site, ponds and seminatural terrestrial habitats were built in the course of the protection program and *H. arborea* was first detected in 2001. Even though no bottleneck could be detected in the other populations, this does not signify that these populations never underwent a large population decline, but the method utilised cannot tell about population reductions that took place more than 0.2-4.0 N_e generations ago. The knowledge and the significance of bottlenecks and founder effects should be deepened by further investigation in the Reuss valley population since they are thought to affect the parameters for genetic diversity.

Significance for protections efforts

Despite the constraints the results concerning heterozygosity excess may have significance for the conservation efforts. First, after a colonization of human-made ponds by *H. arborea* additional immigrating individuals are necessary for a larger gene pool. Second, the results imply no drastic reduction in subpopulations located in canton Aargau (on the contrary new subpopulations are recorded) but at least two in canton Zuerich. The immense protection efforts in canton Aargau seem to prevent drastic reductions of the size of subpopulations and even facilitate new subpopulations. This in turn does not signify that the efforts in canton Zuerich are without effect. But since protection efforts there started later and the subpopulations may struggle with the large geographical distance to the next cluster, the protection activities should be enforced.
The correlations between genetic parameters and chorus count parameters stated in the present study indicate that chorus monitoring is adequate to document the situation of *H. arborea* in the Reuss valley. Since there are indications that not only the chorus size itself but also the relative variation of the chorus size is related to genetics, I recommend not focusing solely on the size of the chorus for statements about the condition of the subpopulations, but also taking into consideration the variability of the subpopulation sizes.

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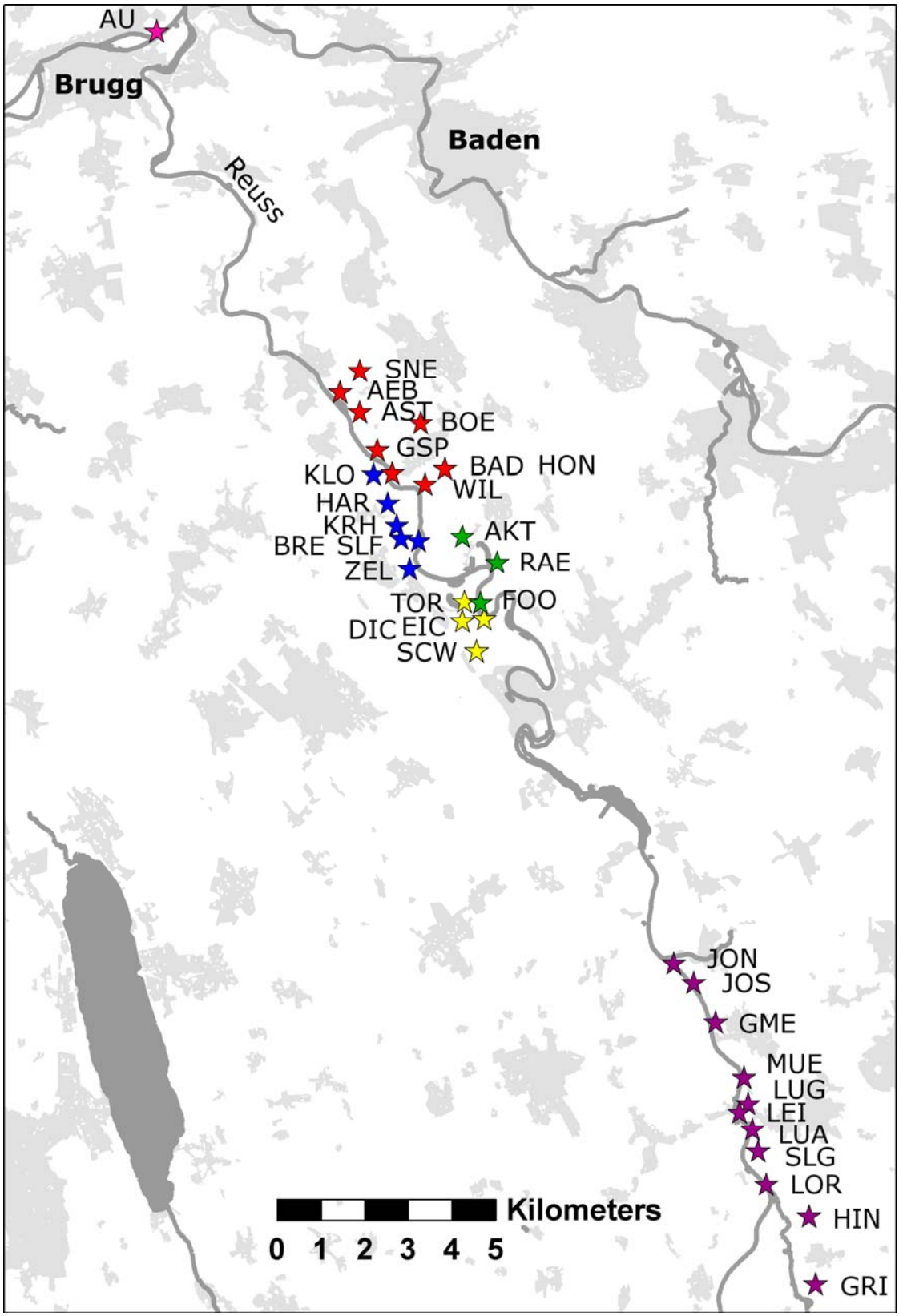
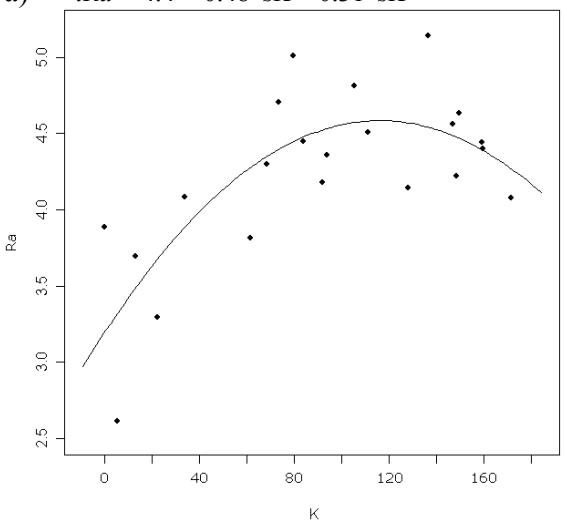
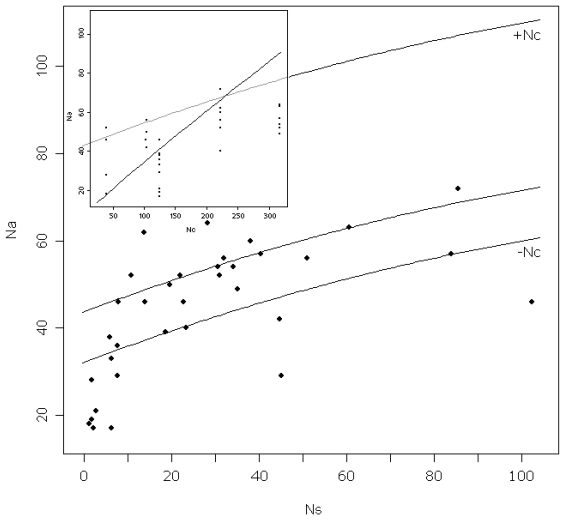


Figure 1. Map of the distribution of *Hyla arborea* in the Reuss valley (GRI: X 659.550, Y 260.070; AU: X 674.650, Y 231.350) along the Reuss river in Switzerland. Each star represents a sampled subpopulation. The six inferred genetic clusters are marked with different colours (for more information see text and Table 2). Populated areas are illustrated in light grey, towns are named.

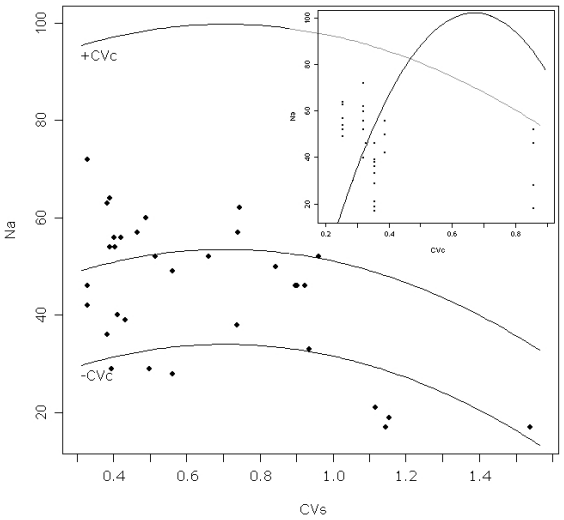
a) $tRa = 4.4 + 0.48*sK - 0.31*sK^2$



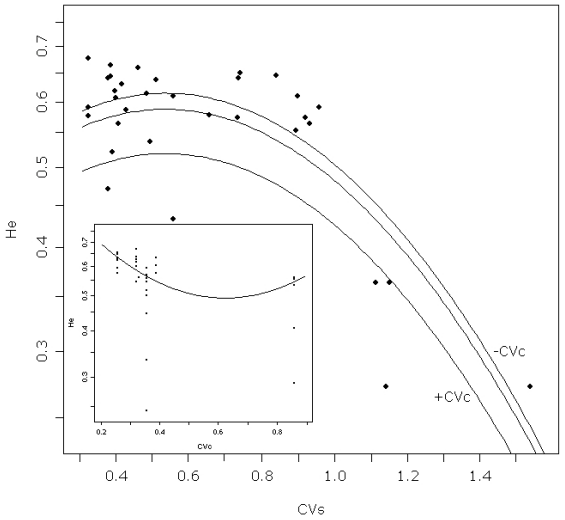
b) $Na = 66.8 + 8.25*sNs - 0.65*sNs^2$
$Na = 66.8 + 25.53*sNc - 0.59*sNc^2$



c) $Na = 66.8 + 0.99 *sCVs - 2.62*sCVs^2$
$Na = 66.8 + 51.77*sCVc - 18.92*sCVc^2$



d) $tHe = -1 - 0.11*sCVs - 0.15*sCVs^2$
$tHe = -1 - 0.26*sCVc + 0.11*sCVc^2$



e) $tF_{IS} = 0.05 - 0.1*sCVs - 0.35*sCVs^2$
$tF_{IS} = 0.05 - 0.1*sCVc + 0.06*sCVc^2$

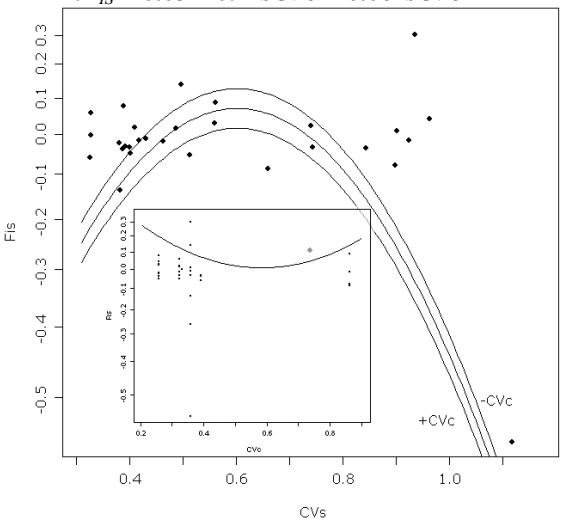


Figure 2. Correlation between chorus count parameters and genetic parameters of the *Hyla arborea* metapopulation in the Reuss valley in Switzerland. (a) allelic richness and connectivity, (b) number of alleles and chorus size (c) number of alleles and coefficient of variation of the chorus size, (d) expected heterozygosity and the coefficient of variation of the chorus size, (e) the inbreeding coefficient and the coefficient of variation of the chorus size. The small graphs show the corresponding correlation between the genetic parameters and the chorus count parameters at the cluster level.

Table 1. The four groups of independent parameters (s: subpopulation, c: genetic cluster). Within each group AIC_i and $AICw_i$ was calculated for each listed combination of parameters and squared parameters.

Chorus size	Population trend	Coefficient of variation of the chorus size	Connectivity
Ns	Ts	CVs	K
Nc	Tc	CVc	$K+K^2$
$Ns+Nc$	$Ts+Tc$	$CVs+CVc$	
$Ns+Ns^2$	$Ts+Ts^2$	$CVs+CVs^2$	
$Nc+Nc^2$	$Tc+Tc^2$	$CVc+CVc^2$	
$Ns+Nc+Ns^2$	$Ts+Tc+Ts^2$	$CVs+CVc+CVs^2$	
$Ns+Nc+Nc^2$	$Ts+Tc+Tc^2$	$CVs+CVc+CVc^2$	
$Ns+Nc+Ns^2+Nc^2$	$Ts+Tc+Ts^2+Tc^2$	$CVs+CVc+CVs^2+CVc^2$	

Table 2. Population genetic data and chorus count data (n: number of sampled individuals for genetic analysis, AG: Aargau, ZH: Zürich).

Subpopulation		n	*Ra*	*Na*	*He*	*Fis*	*Ns*	*Ts*	*CVs*	*K*		*Nc*	*Tc*	*CVc*
AU	Auschachen	18	3.88	46	0.58	0.00	102.25	-0.05	0.33	0.02	PI	102.25	-0.05	0.33
SNE	Schneeschmelze	21	5.01	64	0.64	0.08	28.00	0.13	0.39	79.24	R	315.50	0.02	0.26
AEB	Aebereich	33	4.70	63	0.64	-0.02	60.38	0.08	0.38	73.32				
AST	Aegerten ST	23	4.51	57	0.64	0.02	40.25	-0.01	0.74	110.54				
BOE	Boesimoos	36	4.45	57	0.66	-0.02	83.75	0.03	0.46	83.45				
GSP	Gspiss	17	4.63	54	0.67	-0.04	30.38	-0.05	0.39	148.62				
BAD	Bachdole	22	4.08	49	0.61	0.03	35.00	-0.04	0.56	170.70				
HON	Honert	27	4.15	52	0.59	0.04	10.75	0.11	0.96	127.38				
WIL	Wildenau	23	4.22	54	0.61	-0.05	34.00	0.00	0.40	147.75				
KLO	Klosteraecker	31	4.40	56	0.63	-0.02	31.88	0.10	0.42	158.52	B	220.88	0.03	0.32
HAR	Hard	30	5.14	72	0.68	0.06	85.25	0.07	0.33	135.75				
KRH	Kraehhuebel	31	4.44	60	0.62	0.02	37.88	0.03	0.49	158.20				
BRE	Breiti	13	4.56	52	0.64	-0.05	30.75	0.08	0.51	146.10				
SLF	Schlaufe	4		40	0.57	0.02	23.25	0.03	0.41	143.72				
ZEL	Zelgli	21	4.81	62	0.65	-0.03	13.57	0.20	0.74	104.82				
AKT	Aegerten KT	31	4.36	56	0.62	-0.04	50.86	0.07	0.40	93.39	G	103.75	0.04	0.39
RAE	Raegelrain	6		42	0.59	-0.06	44.63	0.11	0.33	61.84				
FOO	Foort	17	4.30	50	0.65	-0.04	19.50	0.06	0.84	68.03				
DIC	Dickhoelzli	26	3.81	46	0.55	-0.08	13.83	0.19	0.90	61.14	Y	38.25	0.05	0.86
TOR	Tote Reuss	11	4.18	46	0.57	-0.02	7.80	0.27	0.92	91.41				
SCW	Schwand	31	4.08	52	0.58	-0.09	21.88	0.14	0.66	33.67				
EIC	Eichholz	3		28	0.43	0.09	1.60	1.25	0.56	69.26				
JON	Jonen N	2		21	0.36	-0.56	2.63	0.26	1.11	10.62	P	123.88	0.02	0.35
JOS	Jonen S	1		17	0.27		2.13	-0.05	1.54	19.01				
GME	Gmeimatt	23	2.61	29	0.54	0.14	45.00	0.04	0.49	5.53				
MUE	Muelibach	1		19	0.36		1.71	0.30	1.15	24.79				
LUG	Lunnergrien	5		33	0.57	0.30	6.14	-0.19	0.93	28.68				
LEI	Leiloch	4		29	0.52	-0.03	7.50	-0.52	0.39	28.65				
LUA	Lunnerallmend	1		17	0.27		6.25	-0.02	1.14	29.57				
SLG	Schlaenggen	27	3.29	39	0.59	-0.01	18.50	0.09	0.43	22.41				
LOR	Lorzespitz	27	3.69	46	0.61	0.01	22.63	0.07	0.90	13.37				
HIN	Hinterfeld	8		38	0.57	-0.26	5.75	0.15	0.74	11.97				
GRI	Grischhei	7		36	0.47	-0.14	7.57	0.48	0.38	3.86				

Table 3. Results of model selection° and the model averaging*, including upper and lower 95% confidence level. Blank cells indicate that the according independent parameters did not exhibit an $AICw_i$>0.05 whereby they have not been comprised in the final model presented in this table.

	Inter-cept	*sNp*	*sNc*	*sNp* ^{2}	*sNc* ^{2}	*sCVp*	*sCVc*	*sCVp* ^{2}	*sCVc* ^{2}	*sK*	*sK* ^{2}
° Ra	4.40									0.48	-0.31
lowCL	4.16									0.39	-0.41
upCL	4.63									0.58	-0.22
* Na	66.80	8.25	25.53	-0.65 •	-0.59 •	0.99 •	51.77	-2.62	-18.92		
lowCL	50.27	3.55	13.78	-3.05	-2.68	-2.50	19.72	-4.57	-31.61		
upCL	83.33	12.96	37.28	1.74	1.51	4.48	83.83	-0.67	-6.23		
° tHe	-1.00					-0.11	-0.26	-0.15	0.11		
lowCL	-1.20					-0.17	-0.40	-0.19	0.04		
upCL	-0.82					-0.05	-0.12	-0.11	0.19		
° tFis	0.05					-0.10 •	-0.10 •	-0.34	0.06 •		
lowCL	-0.35					-0.21	-0.35	-0.51	-0.08		
upCL	0.45					0.01	0.14	-0.16	0.19		

Appendix

A. Results of the model selection

Table A. Results of the model selection. Selected models ($AICw_i$>0.05) are highlighted in grey.

Allelic richness

Models	AIC_i	$AICw_i$	AICw
$K+K^2$	-39.97	0.9687	**0.9687**
K	-32.38	0.0218	
Nc	-28.62	0.0033	
$Ns+Nc$	-26.74	0.0013	
$Nc+Nc^2$	-26.62	0.0012	
$Ns+Nc+Ns^2$	-25.30	0.0006	
$Ns+Nc+Nc^2$	-24.75	0.0005	
$CVc+CVc^2$	-24.66	0.0005	
CVc	-23.72	0.0003	
CVs	-23.31	0.0002	
$Ns+Nc+Ns^2+Nc^2$	-23.30	0.0002	
$CVs+CVc+CVs^2$	-22.98	0.0002	
Ns	-22.94	0.0002	
Tc	-22.59	0.0002	
Ts	-22.54	0.0002	
$CVs+CVc$	-21.94	0.0001	
$CVs+CVs^2$	-21.59	0.0001	
$CVs+CVc+CVs^2+CVc^2$	-21.25	0.0001	
$Tc+Tc^2$	-21.15	0.0001	
$Ns+Ns^2$	-21.07	0.0001	
$Ts+Tc$	-20.60	0.0001	
$Ts+Ts^2$	-20.54	0.0001	
$CVs+CVc+CVc^2$	-20.22	0.0000	
$Ts+Tc+Ts^2$	-19.30	0.0000	
$Ts+Tc+Tc^2$	-18.60	0.0000	
$Ts+Tc+Ts^2+Tc^2$	-17.45	0.0000	

Number of alleles

Models	AIC_i	$AICw_i$	AICw
$Ns+Nc+Ns^2$	160.06	0.2817	0.2817
$Ns+Nc+Ns^2+Nc^2$	160.62	0.2120	0.4937
$CVs+CVc+CVs^2$	161.21	0.1583	0.6520
$CVs+CVc+CVs^2+CVc^2$	161.27	0.1538	0.8058
$Ns+Ns^2$	161.78	0.1188	**0.9246**
$Ns+Nc$	165.18	0.0217	
$Ns+Nc+Nc^2$	166.00	0.0144	
$CVs+CVs^2$	166.71	0.0101	
CVs	166.84	0.0095	
$CVs+CVc+CVc^2$	167.79	0.0059	
$K+K^2$	168.08	0.0051	
$CVs+CVc$	168.62	0.0039	
K	168.84	0.0035	
Ns	172.37	0.0006	
Nc	173.06	0.0004	
$Nc+Nc^2$	174.42	0.0002	
$CVc+CVc^2$	177.54	0.0000	
$Ts+Ts^2$	178.37	0.0000	
Ts	179.20	0.0000	
$Ts+Tc+Tc^2$	179.92	0.0000	
$Ts+Tc$	180.76	0.0000	
$Ts+Tc+Ts^2+Tc^2$	181.38	0.0000	
$Ts+Tc+Ts^2$	182.46	0.0000	
CVc	184.65	0.0000	
Tc	187.35	0.0000	
$Tc+Tc^2$	189.28	0.0000	

Table A. (Continuance). Results of the model selection. Selected models ($AICw_i$>0.05) are highlighted in grey.

Expected heterozygosity

Models	AIC_i	$AICw_i$	AICw
CVs+CVc+CVs²+CVc²	-32.47	0.3729	0.3729
CVs+CVs²	-32.30	0.3418	0.7147
CVs+CVc+CVc²	-31.90	0.2802	**0.9949**
CVs	-22.01	0.0020	
CVs+CVc+CVs²	-21.48	0.0015	
CVs+CVc	-20.01	0.0007	
Ns+Ns²	-19.32	0.0005	
Ns+Nc+Ns²	-17.71	0.0002	
Ns+Nc+Ns²+Nc²	-15.97	0.0001	
Ns+Nc	-9.90	0.0000	
Ns+Nc+Nc²	-8.10	0.0000	
Ns	-7.92	0.0000	
K	-7.44	0.0000	
K+K²	-6.11	0.0000	
Nc	-5.02	0.0000	
Nc+Nc²	-3.11	0.0000	
Ts	-2.04	0.0000	
CVc+CVc²	-1.28	0.0000	
Ts+Ts²	-0.61	0.0000	
Ts+Tc	-0.54	0.0000	
CVc	0.42	0.0000	
Ts+Tc+Ts²	0.80	0.0000	
Ts+Tc+Tc²	0.88	0.0000	
Ts+Tc+Ts²+Tc²	2.08	0.0000	
Tc	2.90	0.0000	
Tc+Tc²	4.84	0.0000	

Observed heterozygosity

Models	AIC_i	$AICw_i$	AICw
K	-52.98	0.1616	0.1616
Ts+Ts²	-52.39	0.1204	0.2820
Nc	-52.38	0.1199	0.4018
K+K²	-51.40	0.0732	0.4750
CVc	-51.09	0.0628	0.5378
Ns	-50.92	0.0577	0.5955
Ts+Tc+Tc²	-50.77	0.0534	0.6489
Ns+Nc	-50.65	0.0503	0.6992
Nc+Nc²	-50.46	0.0459	0.7450
Ns+Ns²	-50.08	0.0378	0.7828
Tc	-49.86	0.0339	0.8167
CVc+CVc²	-49.47	0.0279	0.8446
Ns+Nc+Ns²	-48.96	0.0217	0.8663
Ts+Tc+Ts²+Tc²	-48.77	0.0197	0.8859
Ns+Nc+Nc²	-48.71	0.0191	**0.9051**
CVs	-48.51	0.0173	
Tc+Tc²	-48.14	0.0143	
Ts	-48.10	0.0140	
CVs+CVc	-47.84	0.0124	
Ns+Nc+Ns²+Nc²	-47.03	0.0082	
CVs+CVs²	-46.67	0.0069	
CVs+CVc+CVc²	-46.45	0.0062	
Ts+Tc	-46.43	0.0061	
CVs+CVc+CVs²	-45.97	0.0049	
Ts+Tc+Ts²	-44.51	0.0023	
CVs+CVc+CVs²+CVc²	-44.50	0.0023	

Table A. (Continuance). Results of the model selection. Selected models ($AICw_i$>0.05) are highlighted in grey.

Inbreeding coefficient

Models	AIC_i	$AICw_i$	AIC
$CVs+CVs^2$	-69.95	0.5511	0.5511
$CVs+CVc+CVs^2$	-67.96	0.2038	0.7549
$CVs+CVc+CVs^2+CVc^2$	-66.36	0.0915	0.8465
CVs	-63.82	0.0258	0.8723
K	-62.67	0.0145	0.8868
Ns	-62.34	0.0123	0.8990
$CVs+CVc$	-62.07	0.0107	**0.9098**
$Ns+Ns^2$	-61.99	0.0103	
$K+K\text{^}2$	-61.68	0.0088	
$Ts+Ts^2$	-61.64	0.0087	
Nc	-61.42	0.0078	
$CVs+CVc+CVc^2$	-61.24	0.0071	
Ts	-61.11	0.0066	
CVc	-60.64	0.0052	
Tc	-60.62	0.0052	
$Ns+Nc$	-60.61	0.0052	
$Ns+Nc+Ns^2$	-59.99	0.0038	
$CVc+CVc^2$	-59.84	0.0035	
$Ts+Tc+Ts^2$	-59.77	0.0034	
$Nc+Nc^2$	-59.76	0.0034	
$Ts+Tc$	-59.20	0.0026	
$Ns+Nc+Nc^2$	-59.07	0.0024	
$Tc+Tc^2$	-58.71	0.0020	
$Ns+Nc+Ns^2+Nc^2$	-58.52	0.0018	
$Ts+Tc+Ts^2+Tc^2$	-57.99	0.0014	
$Ts+Tc+Tc^2$	-57.52	0.0011	

B. Model averaging

Table B. Model averaging for Na. Blank cells occur if the corresponding independent parameter in not comprised in the combined model.

			Inter.	*Ns*	*Nc*	Ns^2	Nc^2	*CVs*	*CVc*	CVs^2	CVc^2	$AICw_i$
Combined models	$CVs+CVc+CVs^2$	$Ns+Ns^2$	51.66	13.81		-4.17		1.55	0.22	-3.23		0.0006
	$CVs+CVc+CVs^2$	$Ns+Nc+Ns^2$	49.43	10.13	8.13	-2.44		0.74	5.82	-2.48		0.0597
	$CVs+CVc+CVs^2$	$Ns+Nc+Ns^2+Nc^2$	54.72	10.37	14.43	-2.24	-5.63	1.17	11.64	-2.52		0.1119
	$CVs+CVc+CVs^2+CVc^2$	$Ns+Ns^2$	43.74	11.71		-3.51		0.86	-12.29	-2.61	7.00	0.0059
	$CVs+CVc+CVs^2+CVc^2$	$Ns+Nc+Ns^2$	69.84	7.81	28.47	-0.29		0.99	60.86	-2.65	-22.96	0.6006
	$CVs+CVc+CVs^2+CVc^2$	$Ns+Nc+Ns2+Nc^2$	70.03	7.76	28.62	-0.26	0.20	0.98	61.68	-2.65	-23.39	0.2212
Averaged model			66.80	8.25	25.53	-0.65	-0.59	0.99	51.77	-2.62	-18.92	