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Species identification based on a semi-diagnostic marker: evaluation of a simple conchological test for distinguishing blue mussels Mytilus edulis L. and M. trossulus Gould

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Short title: Conchological test for distinguishing *Mytilus edulis* and *M. trossulus*

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Competing Interests

The authors have declared that no competing interests exist.

Abstract

Cryptic and hybridizing species may lack diagnostic taxonomic characters leaving researchers with semi-diagnostic ones. Identification based on such characters is probabilistic, the probability of correct identification depending on the species composition in a mixed population. Here we test the possibilities of applying a semi-diagnostic conchological character for distinguishing two cryptic species of blue mussels, *Mytilus edulis* and *M. trossulus*. These ecologically, stratigraphically and economically important molluscs co-occur and hybridize in many areas of the North Atlantic and the neighboring Arctic. Any cues for distinguishing them in sympatry without genotyping would save much research effort. Recently these species have been shown to statistically differ in the White Sea, where a simple character of the shell was used to distinguish two mussel morphotypes. In this paper, we analyzed the associations between morphotypes and species-specific genotypes based on an abundant material from the waters of the Kola Peninsula (White Sea, Barents Sea) and a more limited material from Norway, the Baltic Sea, Scotland and the Gulf of Maine. The performance of the "morphotype test" for species identification was formally evaluated using approaches from evidence-based medicine. Interspecific differences in the morphotype frequencies were ubiquitous and unidirectional, but their scale varied geographically (from 65% in the White Sea to 6% in the Baltic Sea). In addition, salinity-related variation of this character within *M. edulis* was revealed in the Arctic Barents Sea. For every studied region, we established relationships between the proportions of the morphotypes in the populations as well as between the proportions of the morphotypes in samples and the probabilities of mussels of different morphotypes being *M. trossulus* and *M. edulis*. We provide recommendations for the application of the morphotype test to mussels from unstudied contact zones and note that they may apply equally well to other taxa identified by semi-diagnostic traits.

Introduction

Blue mussels *Mytilus edulis* and *M. trossulus* are old evolutionary lineages of Pliocene origin [1]. The more common *M. edulis* is thought to be native in the Atlantic, while the originally Pacific *M. trossulus* has colonized the northwest Atlantic in a series of natural and anthropogenic invasions [2–4]. Now these two species co-occur and hybridize in at least six geographical sectors of the North Atlantic and the adjacent Arctic coasts: western Greenland, American coast from the Gulf of Maine to Hudson Bay, northeastern Scotland, western Baltic Sea, western Norway and the coasts of the Kola Peninsula in Russia (White Sea, Barents Sea) ([5] and references therein).

Ever since the existence of *M. trossulus* was recognized by molecular genetic markers [6], the search has been on for reliable morphometric characters allowing one to distinguish it from *M. edulis*. The discreteness of these two species was confirmed in studies employing numerous metric shell traits in a multivariate approach, but no individually diagnostic characters have been found [7–10]. Therefore *M. edulis* and *M. trossulus* are generally treated as cryptic species and are routinely identified by molecular markers. While multilocus analysis is desirable for an unambitious identification of species and their hybrids, in practice singular presumably diagnostic markers are usually employed, most often the coding region of the polyphenolic adhesive protein gene (ME 15/16 or Glu-5') [11].

Mytilus edulis and *M. trossulus* are ecologically, economically and stratigraphically important molluscs [12–14]. Apart from their different biogeographic histories, these two species are known or suspected to differ in life traits, ecological requirements and properties as biomonitoring and aquaculture objects [15–18]. An illustrative example is the harm associated with *M. trossulus* invasion on longline aquaculture designed for *M. edulis*. A cryptic presence of *M. trossulus* in *M. edulis* plantations in Loch Etive (Scotland) in the 2000s resulted in significant production losses because *M. trossulus* had lower

consumer properties and shells too fragile for harvesting and grading [19,20]. Considerable differences between species were also found in Canadian aquaculture [8,21], where the commercial value of *M. trossulus* was estimated to be 1.7 times less than that of *M. edulis* [8]. The difficulty of identifying *M. edulis* and *M. trossulus* by the shells is frustrating, and any cue for distinguishing these species in sympatry without genotyping would be a welcome addition to the toolkit of mussel studies.

We have recently discovered that *M. edulis* and *M. trossulus* in the White Sea differ by a simple conchological trait: the presence or absence of an uninterrupted prismatic strip under the ligament on the inner side of the shell. This strip is found in 74% of *M. trossulus*-like mussels (i.e. mussels with multilocus genotypes dominated by *M. trossulus* genes; this group mostly consists of purebreds but also includes some hybrids), while 96% of *M. edulis*-like mussels lack this character [16,22]. Hence we denote the mussels that bear the strip as the T-morphotype and those that lack this strip, as the E-morphotype.

This finding raises two questions. The first is how to apply this marker for individual and population assignment correctly and efficiently. Species identification is usually based on fixed diagnostic traits, which have a unique state for all individuals of a species. The conchological trait under consideration is not diagnostic but semi-diagnostic, i.e. polymorphic within species but with states distributed in different frequencies across species (see [23]). Since there are strong (70%) differences in the morphotype frequencies between the mussel species in the White Sea, one can fall into a trap of deciding that any T-morphotype mussel from the White Sea can be assigned with a high probability to *M. trossulus* while any E-morphotype mussel can be assigned to *M. edulis*. In fact, however, the probabilities of correct identification depend on the proportions of *M. trossulus* and *M. edulis* in the mixed population under study. Any mussel sampled from a “pure” *M. trossulus* population (an expected T-morphotype frequency $PT = 74\%$) would be *M. trossulus* regardless of the morphotype. By

126 the same token, any mussel sampled from a "pure" *M. edulis* population (*PT* =4%) would be *M. edulis*.
127 At the same time, in a 1:1 mixture of species (expected *PT* = (74+4)/2 = 39%), 95% of the T-
128 morphotypes would be *M. trossulus* (*P(tros|T)* = 0.74x0.5/(0.39) = 0.949), while 79% of the E-
129 morphotypes would be *M. edulis* (*P(edu|E)* = 0.96x0.5/(1-0.39) = 0.787). However, these calculations
130 are valid only if the morphotype frequencies within 'species-specific' genotypes do not vary with the
131 proportions of species in mixed populations ("taxonomic structure of populations" hereinafter).

132 In such a situation, taxonomists may profit from the experience of clinicians. They often have to deal
133 with semi-diagnostic characters since many clinical diagnostic tests employ semi-diagnostic markers. A
134 formal procedure has been developed in evidence-based medicine to evaluate the ability of clinical tests
135 to classify patients as having or not having the target condition relative to the reference standard
136 (e.g. [24]). We suggest that this methodology might be useful for the evaluation of taxonomic tests for
137 cryptic species relative to the species-specific genotype. To emphasize the analogy with the clinical
138 approach, we refer to the procedure of mussel species identification based on the morphotype as the
139 "morphotype test".

140 The second question is whether the basic morphological differences between *M. trossulus* and *M.*
141 *edulis* revealed in the White Sea are a local phenomenon or whether these two species can be
142 distinguished by the morphotype in other populations and contact zones as well. Should the latter prove
143 true, the morphotype test would facilitate local mussel studies in the Atlantic. Since interspecific
144 differences in this particular character were overlooked in previous morphometric studies, which all
145 were based on references from outside of the White Sea [7–10], it remains possible that this difference
146 is indeed valid only in the White Sea. Such a situation could be associated with the unusual
147 environment, featuring a combination a subarctic climate and a relatively low salinity (< 25 ppt —
148 [25]) and/or with the history of the local *M. trossulus*. *M. trossulus* is thought to have invaded the Kola

Peninsula with marine traffic only recently, in the middle of the 20th century, while most other Atlantic populations are probably much older [3].

In this paper we address the above two questions. Firstly we analyze the associations between morphotypes and species-specific genotypes in an abundant material from the waters of the Kola Peninsula and in more limited material from Norway, the Baltic Sea, Scotland and the Gulf of Maine. For the Kola material, we compare populations from the marginal, semi-enclosed White Sea and from the oceanic Barents Sea coasts on the one hand, and populations from the brackish vs saline localities in the Barents Sea on the other hand. The purpose is to see how local geography and salinity (or associated factors) affect morphotype frequencies in populations with similar biogeographic histories existing under similar climatic conditions. Secondly, we formally evaluate the performance of the "morphotype test" for species identification using approaches from evidence-based medicine, and provide practical recommendations for its use for population and individual assessment.

Materials and Methods

Samples. Altogether, we considered 77 mussel samples (total sample size N = 4325, individual sample size N=18-173) representing five geographical contact zones between *M. edulis* and *M. trossulus*: the Gulf of Maine in the northwestern Atlantic (12 samples, N = 428), Loch Etive in northern Scotland (2 populations, N = 160), western Baltic Sea (8 samples, N = 638), Bergen city area in western Norway (5 samples, N = 365) and the coasts of the Kola Peninsula in northern Russia: 24 samples from the White Sea (N=1089) and 26 samples from the Barents Sea (N = 1645). Detailed information about samples and sampling localities is provided in the **S1 Table**.

The Barents Sea samples were taken in the Kola Bay and at the open oceanic coast of the eastern Murman. Based on the salinity in the sampling localities, they were classified into brackish (salinity 5-

30 ppt) and saline (>30 ppt). The first group consisted of nine samples from the freshened top of the Kola Bay and three samples from the open coast. The second group consisted of eight samples from the mouth of the Kola Bay and six samples from the open coast (**Fig 1**). As for the samples from the other contact zones, all American samples and two out of five Norwegian samples were from saline habitats, while all the others were from brackish habitats. Salinity conditions in the sampling localities were either taken from the literature [26–31] or, in case of the few American and the Barents Sea open coast localities, predicted based on the presence or absence of large rivers nearby.

Fig 1. Map of the study area and variation in shell morphotype frequencies. The bottom panel (maps G-K) shows five geographical contact zones between *M. edulis* and *M. trossulus*, maps in the upper panel (A - F) - other studied areas. Pins depict sampling sites. Pie diagrams depict proportions of T-morphotypes (black sector) and E-morphotypes (white sector) in *M. trossulus* (diagrams with a red border) and *M. edulis* (those with a blue border) in combined samples from particular regions. If the data on salinity in sampling localities are available and considered in the analyses, it is indicated by the color of pins (light green – brackish, dark green – saline, white – salinity is unknown) and the proportions of the T-morphotypes in combined samples from brackish and saline localities are presented separately in diagrams placed on light and dark green background, respectively. Source data are given in **S1 Table** and **S2 Table**.

In addition to the samples taken in the five contact zones, we identified the morphotypes in 27 samples (total sample size N=912, individual sample size N=12-76) of supposedly pure blue mussel species from distant localities: *M. trossulus* from Passamaquoddy Bay and *M. edulis* from the Gulf of Saint Lawrence in eastern Canada, *M. trossulus* from the northern Baltic Sea, from Puget Sound in eastern Pacific and from multiple areas of western Pacific, *M. edulis* from southwestern Greenland, from the Long Island Sound and Cape Cod in the eastern USA, and from saline and brackish localities in Europe

194 and in the southwestern Barents Sea (**Fig 1**, **S2 Table**). Information about the species identity of
195 regional populations and salinity conditions in sampling localities was taken from the literature.
196 Taxonomic affinities of mussels from Canada and from western Norway, where both species could be
197 expected, were confirmed genetically (see **S2 Table** for details).

198 **Genetic characters.** A part of the material, from the contact zones, was genotyped in previous studies
199 (8 of 12 American samples: [30]; 6 of 8 Baltic samples: [3]; 2 of 5 Norwegian samples: [3]; 1 of 26 the
200 Barents Sea samples: [3]; all the White Sea samples: [16]). The other samples were collected and
201 genotyped specially for this study (see **S1 Table**). The Gulf of Maine mussels, both from previous and
202 new material, were genotyped using 109 260 SNPs (single nucleotide markers) as described in [30]
203 (including some material from [33]). The mussels from the other areas were genotyped using various
204 sets of allozyme loci which, as a rule, included the four nearly diagnostic loci Est-D, Gpi, Pgm and
205 Odh, which individually show 70–95% allele frequency differences between *M. edulis* and *M.*
206 *trossulus*. Other new samples were genotyped by Est-D, Gpi, Pgm and Odh as in [16]. For seven
207 samples from the Barents Sea the data on only three loci (Est-D, Gpi and Pgm) were available (see **S1**
208 **Table**). The SNP data set and each of the four regional 4-locus allozyme sets (from the Baltic, Norway,
209 Scotland and Russia) were analyzed separately using STRUCTURE or fastSTRUCTURE software
210 ([34], settings as in [16] and [30]). The obtained q-values are defined as estimates of the proportion of
211 *M. trossulus* genes in individual genotypes (proportion of *M. edulis* genes is therefore 1-q). The
212 material from Russia was also analyzed by three loci (all but Odh) to show that the exclusion of Odh
213 did not affect the inference (data not shown). The mussels were classified into two categories by their
214 q-values: genotypes dominated by *M. trossulus* genes (q-value > 0.5) and those by *M. edulis* genes (q-
215 value ≤ 0.5). For ease of presentation, these categories will be referred to as "*M. trossulus*" and " *M.*
216 *edulis*" genotypes although each includes both purebreds and hybrids. Only the results of classification

217 into *M. trossulus* and *M. edulis* are considered here. More detailed analyses of the hybrid zones studied
218 have been presented earlier [3,5,16,30,32].

219 **Morphological characters**. Data on the White Sea samples were taken from [16] and the other
220 samples were processed accordingly. We measured the maximum length of each shell to the nearest 0.1
221 mm with electronic calipers and investigated the inner surface of the valves under a dissecting stereo-
222 microscope. A mussel was classified as a T- or an E-morphotype based on, respectively, presence or
223 absence of an uninterrupted strip of the prismatic layer under the ligament on the inner side of the shell.
224 To note, this character was called "the dark strip" in previous papers [16,22] since mussels from the
225 White Sea usually possess a dark prismatic layer, and T-morphotypes were illustrated with photos
226 where the strip was both dark and quite wide. The new material has revealed some geographical
227 variation in the coloration and width of the strip, however. We specify the definitions of the two
228 morphotypes in the Results and provide more illustrations in the in the supporting information.

229 **Predictive values**. For each sample we calculated the frequencies of *M. trossulus* (*Ptros*) and of T-
230 morphotypes (*PT*) and four indices reflecting the strength of association between genotypes and
231 morphotypes: *P(T|tros)* – the proportion of T-morphotypes among *M. trossulus*, *P(E|edu)* – the
232 proportion of E-morphotypes among *M. edulis* (for practical reasons we used *P(T|edu)*=1- *P(E|edu)*,
233 the proportion of T-morphotypes among *M. edulis*), *P(tros|T)* – the proportion of *M. trossulus* among
234 T-morphotypes, *P(edu|E)* – the proportion of *M. edulis* among E-morphotypes. *P(tros|T)* and *P(edu|E)*
235 are the key indices because they show, respectively, how likely it is that a randomly taken T-
236 morphotype mussel is *M. trossulus* and a randomly taken E-morphotype mussel, *M. edulis*.

237 Here we would like to offer an analogy between the indices used in our study and those used in clinical
238 medicine for evaluating the performance of diagnostic tests. If we consider *M. edulis* as a "healthy"

239 mussel and *M. trossulus* as a “sick” mussel (which is not so far-fetching considering the threat
240 presented by *M. trossulus* to the Scottish aquaculture, [19]), then our terms have the following medical
241 equivalents [24]: *Ptros* is *prevalence*, *P(T|tros)* is *sensitivity*, *P(E|edu)* is *specificity*, *P(tros|T)* is
242 *positive predictive value* and *P(edu|E)* is *negative predictive value* of the morphotype test.

243 As with clinical tests where disease markers are not 100% sensitive, the positive and negative
244 predictive values will depend on the prevalence, i.e. the frequency of the species (or disease) of interest
245 in the material [24]. With increasing *Ptros*, *P(tros|T)* will gradually increase from 0 in pure populations
246 of *M. edulis* to 1 in pure populations of *M. trossulus*, while *P(edu|E)* will show an opposite
247 relationship. For the test to be meaningful, predictive values should be >0.5 since a predictive value of
248 0.5 indicates a random association between the genotype and the morphotype. Assuming that
249 sensitivity and specificity do not depend on the prevalence (though this assumption may be violated,
250 see below), predictive values could be directly predicted basing on the *Ptros* in a sample and the known
251 sensitivity and specificity using the formulas:

252

253 $$P(tros \mid T) = \frac{Ptros \cdot P(T \mid tros)}{Ptros \cdot P(T \mid tros) + (1 - Ptros) \cdot P(T \mid edu)}$$ [Eq 1]

254 $$P(edu \mid E) = \frac{(1 - Ptros) \cdot (1 - P(T \mid edu))}{(1 - Ptros) \cdot (1 - P(T \mid edu)) + Ptros \cdot (1 - P(T \mid edu))}$$ [Eq 2]

255 The prevalence (*Ptros*) in turn could be predicted based on *P(T|edu)*, *P(T|tros)* and *PT* in a sample:

256 $$Ptros = \frac{PT - P(T \mid edu)}{P(T \mid tros) - P(T \mid edu)}$$ [Eq 3]

Statistical analyses. The following analyses were made using the data from the contact zones. Firstly, we studied variation of *PT*, *P(T|tros)*, *P(T|edu)*, *P(tros|T)*, *P(edu|E)* as functions of *Ptros* within and between sample sets representing A) the White Sea (sample set *WS*) and saline (set *BH*) and brackish (set *BL*) water localities in the Barents Sea (Section "Associations between morphotypes and genotypes around the Kola Peninsula"), B) different geographical contact zones between species. Whenever possible, formulas describing empirical relationships between *Ptros* and *PT* and between positive (*P(tros|T)*) and negative (*P(edu|E)*) predictive values and *Ptros* were derived on the basis of regression analysis (Section "Associations between morphotypes and genotypes around the Atlantic"). Secondly, we analyzed genotype-specific associations between morphotypes and the shell size in order to verify the hypothesis that morphological variation under consideration is not related to mussel size (Section "Associations between morphotypes and shell size"). Finally, we tested how well *Ptros*, *P(edu|E)* and *P(tros|T)* could be predicted using formulas Eq.1-3 and the data on the morphotype proportions among species (*P(T|tros)*, *P(T|edu)*) in a few genotyped samples. We concede that the assumption that sensitivity and specificity do not depend on the prevalence can be violated in the morphotype test, as it often is in clinical tests [36] Therefore we focused on finding out which samples were better suited for prediction on the basis of Eq.1-3: the most mixed samples (*Ptros*~0.5) or the combination of the two most pure samples of each species. The samples identified in this way as best suited for prediction can be used as "calibrating" ones (Section "Prediction of taxonomic structure of populations and predictive values of the morphotype test based on probability calculators").

All statistical analyses were performed with functions of R3.6.1 statistical programming language [37]. We used generalized linear (mixed) models, GL(M)Ms, with binomial distribution and a logit link-function. All GLM models were constructed with glm() function from the package "stats" [37] whereas

GLMM were fitted with glmer() function from the package "lme4" [38]. The validity of each model was checked by visual analysis of residual plots and the assessment of the overdispersion presence.

The goodness of fit for the models was assessed by means of pseudo-R2 (Nakagawa and Schielzeth 2013) using the function r.squaredGLMM() from the package "MuMIn" [39]. To assess the role of random factors in GLMM, we compared marginal and conditional pseudo R2 [40]. After the model parameters were estimated, we visualized them by means of regression lines with corresponding 95% confidence intervals.

Associations between morphotypes and genotypes around the Kola Peninsula

The following three regression models were fitted for the data

Model 1: Morphotype proportions (*PT*) as a function of taxonomic structure of mussel populations (*Ptros*). All mussels with a T-morphotype were coded as 1 and all mussels with an E-morphotype were coded as 0. These data were used as a dependent variable, which was regressed against *Ptros* (continuous predictor) and *Set* (discrete predictor with three levels) and interaction between them.

Model 2: Morphotype proportions among species (*P(T|tros)*, *P(T|edu)*) as a function of taxonomic structure of populations (*Ptros*). The dependent variable was coded as in Model 1 and modeled as a function of *Ptros*, *Set*, *Species* (a discrete predictor with two levels) and interaction between terms. The sample was included into the model as a random factor influencing the model intercept.

Model 3: Correctness of species identification (*P(tros|T)* and *P(edu|E)*) as a function of taxonomic structure of populations. The dependent variable was coded as 1 if *M. trossulus* was represented by a T-morphotype or *M. edulis* was represented by an E-morphotype and as 0 in the other cases. The set of predictors for the model was as follows: *Ptros*, *Morphotype* (discrete predictor with two levels), *Set* and

interaction between terms. The sample was included into the model as a random factor influencing the model intercept.

To check whether it is possible to pool some of the geographical sets to construct a more general model without losing information, we constructed three complex data sets with different pairing combinations of *WS*, *BL* and *BH*: (*WSBL*) and *BH*; (*WSBH*) and *BL*; (*BLBH*) and *WS*. We did not consider a full combination of sets since in such a case the factor "*Set*" would be discarded from the model. We applied the structure of Model 3 to these new recombined datasets. Then we compared AICs of these new models with AIC of Model 3 based on non-pooled data. If AIC of a new model was less than the AIC of the initial one, we considered this as a basis for pooling of the corresponding sample sets.

Associations between morphotypes and genotypes around the Atlantic. Five sample sets were considered, representing the Gulf of Maine (*GOM*), the Baltic Sea (*BALT*), western Norway (*NORW*), saline Barents Sea (*BH*) and the White Sea combined with the brackish Barents Sea (*WSBL*, sets *WS* and *BL* were pooled since there pooling did not affect the inference, see Results). Scotland (*SCOT*) was not included in regression analyses because it was represented by only two samples. Three models were constructed:

Model 4. Taxonomic structure (*Ptros*) as a function of morphotype frequencies in populations (*PT*). The dependent variable was coded as in Model 1 and modeled as a function of *PT* (continuous predictor), *Set* and interaction between them. We modeled *Ptros* vs. *PT* but not vice versa, as in the previous analysis, in order to use this model as a reference for the "*Ptros* by *PT* calculator" (see below).

Model 5. Morphotype proportions among species (*P(T|tros)*, *P(T|edu)*) as a function of taxonomic structure of populations (*Ptros*). The model was constructed analogously to Model 2.

Model 6. Correctness of species identification (*P(tros|T)* and *P(edu|E)*) as a function of taxonomic structure of populations (*Ptros*). The model was constructed analogously to Model 3.

Associations between morphotypes and shell size. To check the possible association of morphotypes with size we undertook the following two analyses. Firstly, we constructed a set of logistic regression models for each available species-specific genotype (i.e. *M. edulis* or *M. trossulus*) from each sample. The probability of the presence of the T-morphotype was a dependent variable and mussel size was a predictor in these models. Only cases where slope-terms of the models were statistically significant ($p < 0.05$) after Hochberg's correction for multiple testing [41] were considered. Secondly, we checked the presence of any patterns in residuals from Model 6 (i.e. the main model designed to predict the probability of correct identification of an individual mussel by its morphotype) as a function of mussel size.

Prediction of taxonomic structure of populations and predictive values of the morphotype test based on probability calculators. We applied Eq. 1-3 to predict *Ptros*, *P(edu|E)* and *P(tros|T)* for samples from each data set (*GOM*, *BALT*, *NORW*, *BH*, *WSBL*, *SCOT*) using estimates of morphotype proportions among species (*P(T|tros)*, *P(T|edu)*) obtained from combinations of "calibrating" samples selected based on the results of the following analysis.

We considered all 630 possible pairwise combinations of samples from the *WSBL* dataset. Each pair was characterized by an index of taxonomic similarity between the samples:

$$\text{Delta} = (\text{Ptros1}) \cdot (1 - \text{Ptros2}) + (\text{Ptros2}) \cdot (1 - \text{Ptros1}) \quad \text{[Eq. 4]},$$

where *Ptros1* and *Ptros2* – higher and lower estimates of prevalence in samples. The index varies in a range [0; 1] and takes the value Delta=0 when both samples are pure *M. edulis* (*Ptros1* = *Ptros2* = 0) or pure *M. trossulus* (*Ptros1* = *Ptros2* = 1), Delta=0.5 when both samples are equivalent mixtures of two

species (*Ptros1* = *Ptros2* = 0.5) and Delta=1 when one sample represent pure *M. trossulus* (*Ptros1* = 1) and another pure *M. edulis* (*Ptros2* = 0).

Estimates of *P(T|tros)*, *P(T|edu)* and *PT* were obtained from pooled data on each pair of samples and used for calculation of predicted values of *P(edu|E)* and *P(tros|T)* basing on Eq.1,2 for the range of *Ptros* [0;1] with the step 0.01 ("genotype by morphotype calculator") and predicted values of *Ptros* basing on Eq.3 for the range of *PT* [0;1] with the step 0.01 ("*Ptros* by *PT* calculator"). (Note that dealing with Eq. 1, 2 we assume that *Ptros* is known while in reality it should be assessed using Eq. 3). Values of *P(edu|E)* and *P(tros|T)* obtained by Eq.1, 2 were contrasted with the ones predicted by the Model 6, and values of *Ptros* obtained by Eq. 3 were compared with predictions of Model 4 using correspondence statistics:

$$Goodness = MSS^{-1} \quad \text{[Eq.5]}$$

where MSS – mean sum of squares of difference between predictions of regression model and predictions of equation. Goodness varies (0; infinity) and approaches zero when predictions of models agrees poorly.

Goodness indices for each pair were plotted against the corresponding Delta values and the LOESS regression curve was fitted to find associations between them. Depending on the results of the analyses, we determined which combinations of samples could be used for predictions with best results. Best combinations of samples from each set were used to assess *P(T|edu)* and *P(T|tros)* as parameters of Eq.1-3. Then we calculated predictions from Eq.3 for the range of *PT* and predictions from Eq.1-2 for the range of *Ptros*. These predictions were visually compared with those from regression Model 4 and Model 6, respectively.

Additionally, we tested the "lazy *Ptros* by *PT* calculator" which assumes that samples with the highest and the lowest *PT* in regional sample sets do represent, respectively, pure *M. trossulus* and pure *M. edulis* and that morphotype frequencies in these samples could be directly used as parameters *P(T|tros)* and *P(T|edu)* of Eq.3. *Ptros* values predicted by the calculator for samples from different sets were visually compared with empirical ones.

For illustrative purposes and for the convenience of potential users of the "morphotype test" or any similar semi-diagnostic tests we provide the online "*Ptros* by *PT*" and "genotype by morphotype" probability calculators implementing Eq.1-3 at *https://polydora.shinyapps.io/Calculator/*.

Results

Geographical variation in the manifestation of mussel morphotypes

The studied binary morphological character was previously defined as the "presence/absence of a distinct uninterrupted dark prismatic strip under the ligament", based on material from the White Sea only [16,22]. In the broader material of this study, encompassing different geographical zones, the E-morphotypes looked the same everywhere and conformed to the previous description: the strip was absent, and the nacreous layer totally or partially covered the space under the ligament nympha (**S1 Fig A, C**). However, T-morphotypes showed some variation previously unrecorded in the White Sea. Firstly, most of the populations contained, though rarely, shells in which the nacreous-free strip of the prismatic layer was quite narrow and looked like a stria rather than a strip. Secondly, in all T-morphotypes from the Gulf of Maine and in rare T-morphotypes from the other populations the strip was not dark but pale, as the prismatic layer itself. Such T-morphotypes were difficult to notice by an unaided eye, but could be unambiguously identified with a dissecting microscope, by the presence of a pronounced scar defining the boundary of the nacreous layer under the ligament nympha (**S1 Fig**).

S1 Fig. Variation in the manifestation of mussel morphotypes. A-D. Stereoscopic micrographs of the ligament area of mussel valves. Note that scale bars differ between A-C and D. Strip of the prismatic layer under the ligament nympha is indicated by arrows. A, B. E-morphotypes: the space under the ligament nympha is totally (A) or partially (B) covered by the nacre. C, D. T-morphotypes: a strip of uncovered prismatic layer under the ligament nympha is dark and wide (C; typical of most examined populations) or pale and narrow, recognizable by a scar separating it from the nacreous layer (D; typical of the Gulf of Maine populations). E. External and internal features of the shell valves of *M. trossulus* (right) and *M. edulis* (left) genotypes from the Kola Bay (from sample Sev.17 in S1 table). In most cases T-morphotypes (marked by *) and E-morphotypes could be distinguished by an unaided eye.

Therefore, we amend the description of the T/E morphotype character as follows: the presence/absence of an uninterrupted strip of the prismatic layer under the ligament nympha clearly recognizable by a scar separating the strip from the nacreous layer of the rest of the shell. This description was applicable to all the mussel samples examined in this study.

Associations between morphotypes and genotypes around the Kola Peninsula

Variation patterns of *PT*, *P(T|tros)*, *P(T|edu)*, *P(tros|T)*, *P(edu|E)* as functions of *Ptros* in samples from the White Sea (WS), the brackish Barents Sea (BL) and the saline Barents Sea (BH) are visualized in **Fig 2**. The results of the regression analysis are summarized in **S3 Table**.

Fig 2. Variation of *PT*, *P(T|tros)*, *P(T|edu)*, *P(tros|T)*, *P(edu|E)* as functions of *Ptros* in the White Sea (*WS*), brackish Barents Sea (*BL*) and saline Barents Sea (*BH*). Points – empirical estimates, their size is proportional to sample size (see **S1 table**). Lines – regression model predictions, grey filling – 95% confidence intervals of regressions. (A) Proportions of T-morphotypes (*PT*) (Model 1). (B).

Proportions of T-morphotypes among *M. trossulus* (*P(T|tros)*, filled points) and *M. edulis* (*P(T|edu)*, empty points) (Model 2). (C) Frequencies of *M. trossulus* among T-morphotypes (*P(tros|T)*, filled points) and of *M. edulis* among E-morphotypes (*P(edu|E)*, empty points) (Model 4). Vertical lines on B and C connect subsamples of *M. trossulus* and *M. edulis* from the same samples.

A significant positive association between the proportions of *M. trossulus* (*Ptros*) and the proportions of T-morphotypes (*PT*) in samples was revealed for all the three sample sets (Model 1, **S3 Table**, **Fig 2**). For *WS* and *BL*, the data points were generally scattered around the Y=X line, while the regression lines approached it closely, indicating a high proportionality between *Ptros* and *PT* and an almost straightforward relationship between these values. For *BH*, the data points were scattered above the Y=X line and the regression line lay higher than the regression lines constructed for *WS* and *BL*. This means that in samples with a similar taxonomic structure, the frequencies of T-morphotypes were always higher in the saline localities in the Barents Sea than in the White Sea and the brackish localities in the Barents Sea.

The analysis of the frequencies of T-morphotypes in subsamples of *M. edulis* (*P(T|edu)*) and *M. trossulus* (*P(T|tros)*) against proportions of *M. trossulus* in samples (*Ptros*) revealed the following patterns (Model 2, **S3 Table**, **Fig 2**). There was a universal tendency towards a higher frequency of T-morphotypes among *M. trossulus* than among *M. edulis*. This tendency was quite strong in *WS* and *BL* (expected differences in morphotype frequencies between species about 0.65 for *Ptros*=0.5). In *BH* it was rather weak (expected differences 0.18 for *Ptros*=0.5) due to an increased *P(T|edu)* but significant (confidential intervals for *Ptros*=0.5 did not overlap, **Fig 2**). A positive correlation of *P(T|tros)* and *P(T|edu)* with *Ptros* was found in all the three subsets. This means that with the increasing contribution of *M. trossulus* to the samples the frequencies of T-morphotypes increased both among *M. edulis* and among *M. trossulus*.

The probability of correct identification of *M. trossulus* by the T-morphotype (the frequency of *M. trossulus* among T-morphotypes (*P(tros|T)*) expectedly increased with the increasing *Ptros*, while the probability of correct identification of *M. edulis* by the E-morphotype (*P(edu|E)*) demonstrated an opposite pattern (Model 3, **S3 Table**, **Fig 2**). In the *M. trossulus*-dominated populations, *P(tros|T)* tended to one (any mussel with a T-morphotype is 100% *M. trossulus*), while *P(edu|E)* tended to zero (any mussel with an E-morphotype is 100% *M. trossulus*), and vice versa. In the well-mixed samples (*Ptros* = 0.5) the predictive values for both species were about 0.75-0.85 in WS and BL but only 0.60 - 0.70 in BH (**Fig 2**). It means that the morphotype test has a much lower predictive value in the saline Barents Sea than in the brackish Barents Sea and in the White Sea (the predictive value of 0.5 means a random association between the genotype and the morphotype). It is evident from **Fig 2** that a low predictive value of the test in *BH* is mainly due to a generally high *P(T|edu)*. The statistical analysis indicates that both *P(tros|T)* and *P(edu|E)* predicted by the model were smaller in *BH* than in *WS* and *BL*.

For each of the GLMM models considered (Model 2 and 3), marginal and conditional pseudoR2 were close to each other (**S3 Table**). This indicates that the role of the random factor (*Sample*) as regulator of models was weak, i.e. the reproducibility of the results in different populations was satisfactory.

In comparisons between sets, the regression coefficients did not differ statistically for *WS* and *BL*, while *BH* was always different from *WS* (**S3 Table**). To assess the possibility of pooling the data sets, we compared the AIC of Model 3 (AIC=2175.1) with AICs of three other models based on differently pooled *WS, BL* and *BH* sets. The model based on pooled *WS* and *BL* (*WSBL*) and *BH* showed the lowest AIC (AIC=2172.7). Therefore, in the following analyses we will consider two sets, *WSBL* and *BH*.

Associations between morphotypes and genotypes around the Atlantic

The patterns of *Ptros* variation against *PT* and the patterns of *P(T|tros)*, *P(T|edu)*, *P(tros|T)* and *P(edu|E)* variation against *Ptros* in samples from different geographical zones are visualized in **Fig 3**. The results of the regression analysis are summarized in **S3 Table**. The Scottish material was not included in the regression analyses. Re-analyses of the data from the White and the Barents Seas (*WSBL* and *BH* sets) together with the data from other regions revealed the same patterns as those described above. Again, in all the cases when mixed models were used (Model 5, Model 6, **S3 Table**) the marginal and conditional pseudoR2 were close to each other (**S3 Table**) indicating a weak role of the random factor (*Set*) as regulator of models, i.e. a satisfactory reproducibility of the results from population to population in all the regions.

The proportion of *M. trossulus* in samples (*Ptros*) was positively correlated with the proportion of T-morphotypes (*PT*) in the other sets, as it did in the samples from the White and the Barents Seas. This tendency was significant for all the sets (**Fig 3**; Model 4, **S3 Table**). Otherwise, the patterns of variation were different for different sets. For GOM, the regression line stretched above the Y=X line but close to it, indicating the proportionality between *PT* and *Ptros*. For *BALT*, the regression slope was very steep, and the regression line rapidly diverged from the Y=X line. This was due to the fact that the *PT* range in *BALT* was, unlike the situation in the other sets, very narrow (0-0.4) as compared with the *Ptros* range (~0-1), and the small surplus of T-morphotypes in the samples was accompanied by a strong increase in the *M. trossulus* prevalence. A similar tendency was observed in the scanty material from *NORW*. Both *SCOT* samples fell on the Y=X line. Noteworthy are a few "outlier" samples from *GOM* and *NORW*, in which *PT* was close to zero but *Ptros* was high.

Fig 3. Predictive power of the morphotype test in different contact zones. (A) Dependence of proportion of *M. trossulus* (*Ptros*) on proportion of T-morphotypes (*PT*). Dotted lines are empirical regressions (Model 4). Solid gray lines – predictions of "*Ptros* by *PT* calculator" (Eq. 3). Solid black lines represent Y=X dependence. (B) Probability to find a mussel with a T-morphotype among *M. edulis* (*P(T|edu)*) (empty points), and *M. trossulus* (*P(T|tros)*) (filled points) as a function of *Ptros*. Lines are empirical regressions (Model 5). (C) Probability of correct species identification by the morphotype test: *M. trossulus* by T-morphotype, *P(tros|T)* (filled points) and *M. edulis* by E-morphotype, *P(edu|E)* (empty points) as a function of *Ptros*. Dotted lines are empirical regressions (Model 6). Sold lines – predictions of "genotype by morphotype calculator" for *M. trossulus* (Eq. 1, red line) and *M. edulis* (Eq.2, blue line). On each graph, dots represent the observed proportions in samples, and shaded areas around regression lines – 95% CI of regressions.

While *P(T|edu)* estimates were low everywhere but in *BH*, *P(T|tros)* demonstrated a strong variation among sets and a noticeable variation within some sets (**Fig 3**; Model 5; **S3 Table**). Similarly to *WSBL*, most *M. trossulus* had T-morphotypes in *GOM* and *SCOT* but not in *BALT* and *NORW*. For *Ptros*=0.5, expected differences in the morphotype frequencies between the species were about 0.44 for *GOM*, 0.06 for *BALT* and 0.24 for *NORW*. A significant positive dependence of the frequencies of T-morphotype on *Ptros* among conspecific genotypes, which was so prominent in the White and the Barents Sea, was recorded elsewhere only in *BALT* for *P(T|tros)* (**S3 Table**).

The pattern of dependence of *P(tros|T)* and *P(edu|E)* on *Ptros* in *GOM*, *BALT* and *NORW* (Model 6. **Fig 3**, **S3 Table**) was the same as in the samples from the Kola Peninsula (Model 3. **Fig 2**, **S3 Table**): *P(tros|T)* increased with the increasing *Ptros*, while *P(edu|E)* showed an opposite tendency. To simplify and formalize the comparison, we provide the predictions of Model 6 for equally mixed populations (*Ptros*=0.5) together with their 95% confidence intervals in **Table 1**, where actual

498 proportions of *M. trossulus* among T-morphotypes (*P(T|tros)*) and *M. edulis* among E-morphotypes
499 (*P(T|edu)*) in pooled samples from the respected sets are also provided.

500 **Table 1**. Proportions of *M. trossulus* among T-morphotypes (*P(tros|T)*) and proportions of *M. edulis*
501 among E-morphotypes (*P(edu|E)*) in pooled samples (direct count) and in equally mixed samples
502 (predictions by the regression Model 6) in different sample sets. Low and upper boundaries of 95%
503 confidence intervals are provided for predicted values (in brackets).

	P(edu|E)		*P(tros|T)*	
Set	Ptros=0.5	In the data	Ptros=0.5	In the data
WSBL	0.77 (0.73-0.81)	0.86	0.85 (0.82-0.89)	0.86
BH	0.70 (0.61-0.78)	0.84	0.57 (0.51-0.63)	0.48
GOM	0.66 (0.54-0.77)	0.86	0.86 (0.68-0.95)	0.80
BALT	0.51 (0.44-0.58)	0.46	0.82 (0.58-0.94)	0.93
NORW	0.64 (0.53-0.74)	0.51	0.86 (0.68-0.95)	0.93
SCOT	-	0.90	-	0.96

504

505 For equally mixed populations the predictive values of *P(edu|E)* in *BALT* did not differ significantly
506 from 0.5, which corresponds to an equal probability of correct and incorrect identification. At the same
507 time, the probabilities of correct identification of *M. trossulus* by the T-morphotype in *GOM*, *BALT* and
508 *NORW* were quite high (for the range of *Ptros*≥0.5). In general, the highest predictive values for both
509 species were revealed in *WSBL*.

Using the coefficients of the regression models Model 4 and Model 6 (**S3 Table**), we constructed a set of formulas predicting the taxonomic structure (*Ptros*) and the probability of correct species identification (*P(tros|T)*, *P(edu|E)*) using the morphotype test (**Table 2**). These formulas were further used for the comparison of predictions made with these regression models and the predictions proposed by Eq. 1, 2 and 3.

Table 2. Formulas used for taxonomic and individual assignment using morphotype tests in different sample sets accordingly to the regression model coefficients represented in **S3 Table**.

Region	Model 4	Model 6 E-morphotype	Model 6 T-morphotype
WSBL	$Ptros = \frac{e^{-2.4+5.4PT}}{1+e^{-2.4+5.4PT}}$	$P(edu \mid E) = \frac{e^{3.7-4.9Ptros}}{1+e^{3.7-4.9Ptros}}$	$P(tros \mid T) = \frac{e^{0.2+3.2Ptros}}{1+e^{0.2+3.2Ptros}}$
BH	$Ptros = \frac{e^{-3.9+5.0PT}}{1+e^{-3.9+5.0PT}}$	$P(edu \mid E) = \frac{e^{3.3-4.8Ptros}}{1+e^{3.3-4.8Ptros}}$	$P(tros \mid T) = \frac{e^{-2.1+4.7Ptros}}{1+e^{-2.1+4.7Ptros}}$
GOM	$Ptros = \frac{e^{-2.3+6.2PT}}{1+e^{-2.3+6.2PT}}$	$P(edu \mid E) = \frac{e^{4.7-8.1Ptros}}{1+e^{4.7-8.1Ptros}}$	$P(tros \mid T) = \frac{e^{-0.6+4.9Ptros}}{1+e^{-0.6+4.9Ptros}}$
BALT	$Ptros = \frac{e^{-0.6+11.6PT}}{1+e^{-0.6+11.6PT}}$	$P(edu \mid E) = \frac{e^{2.7-5.4Ptros}}{1+e^{2.7-5.4Ptros}}$	$P(tros \mid T) = \frac{e^{-0.4+3.9Ptros}}{1+e^{-0.4+3.9Ptros}}$
NORW	$Ptros = \frac{e^{-0.5+3.7PT}}{1+e^{-0.5+3.7PT}}$	$P(edu \mid E) = \frac{e^{3.1-5.0Ptros}}{1+e^{3.1-5.0Ptros}}$	$P(tros \mid T) = \frac{e^{-1.5+6.3Ptros}}{1+e^{-1.5+6.3Ptros}}$

Variation in morphotype frequencies between *M. edulis* and *M. trossulus* within and between contact zones revealed in the study is illustrated in **Fig 1**, where the estimates of *P(T|edu)* and *P(T|tros)* in pooled samples from different sets are provided. *P(T|edu)* was 0.53 in the saline Barents Sea (*BH*) and less than 10% in all the other sets. In its turn, *P(T|tros)* was 0.17 in *BALT*, 0.42 in *NORW*, 0.49 in the

522 *GOM* and more than 0.75 in *WSBL* and *SCOT*. *P(T|tros)* estimates in Norway and the Gulf of Maine
523 were much affected by the outlier samples (see above). If we discard these samples, *P(T|tros)* will
524 make up 0.54 in Norway and 0.71 in the Gulf of Maine.

525 **Fig 1** also shows the morphotype frequencies in putatively pure populations of species sampled at a
526 distance from the contact zones. Within the ancestral range of *M. trossulus* in the Pacific, the
527 populations were nearly monomorphic for the T-morphotype. In the Passamaquoddy Bay *P(T|tros)* was
528 0.81, i.e. close to that in most of *M. trossulus* populations in the Gulf of Maine. All reference *M. edulis*
529 populations from temperate areas (Long Island Sound and Cape Cod in western Atlantic, Northern and
530 Norwegian Seas in Europe) were nearly monomorphic for the E-morphotype. At the northeast extreme
531 of the species range in Europe, in the southwestern Barents Sea, *P(T|edu)* varied considerably between
532 the samples, in particular between the samples from brackish (range 0-3%) and saline (0.35-0.70%)
533 localities (see **S2 Table**), as it did along the Barents sea coast of the Kola Peninsula. Increased
534 *P(T|edu)* was also recorded in two northernmost samples from western Atlantic, Greenland (0.66) and
535 the Gulf of Saint Lawrence (0.73).

536 *Associations between morphotypes and shell size*

537 There was no clear statistical relationship between the size and the morphotype of conspecific mussels.
538 At the level of individual samples, the probability of finding a T-morphotype increased with the mussel
539 size (a positive slope-term of the regression) in 16 out of 34 informative comparisons (when species-
540 specific genotypes were both present and polymorphic for morphotypes) for *M. edulis* and in 17 out of
541 43 comparisons for *M. trossulus*. The slope-terms of the regression models were individually
542 significant ($p<0.05$) in four cases for *M. edulis* and in four cases for *M. trossulus*, but only in one case
543 when the correction for multiple testing was applied (sample Berg05, see **S4 table**). We also checked

for the presence of any patterns in residuals from Model 6 as a function of mussel size but none was found.

Prediction of taxonomic structure of populations and predictive values of the morphotype test based on probability calculators

We applied Eq.1 and Eq. 2 ("genotype by morphotype calculator") and Eq. 3 ("*Ptros* by *PT* calculator") using as an input for assessment of equations parameters (*P(T|tros), P(T|edu)*) the data on all possible pairs of samples from *WSBL* and compared the values predicted by these equations with those predicted by regression Models 6 and 4, respectively (**Table2**).

Fig 4 illustrates the goodness of correspondence of the two predictions depending on the genetic constitution of the paired samples as expressed by the Delta index. The best predictions of *Ptros* were obtained when the most dissimilar samples consisting of nearly pure *M. edulis* and *M. trossulus* (Delta >0.75) were taken for Eq.3 calibration. The best predictions of *P(edu|E)* and *P(tros|T)* values were obtained when the most mixed samples (*Ptros* of both samples close to 0.5; range of Delta 0.25-0.5) were taken for Eq.1-2 calibration.

Fig 4. Correspondence between "*Ptros* by *PT* calculator" (Eq. 3, left graph) and "genotype by morphotype calculator" predictions (Eq. 1-2, right graph) and regression Model 6 and Model 4, respectively. Each point corresponds to a unique pair combination of samples from *WSBL* set. OX axis reflects dissimilarity of genetic structure in each pair (Delta) (for pure conspecific samples Delta takes a value of zero, for equally mixed samples – 0.5, for two pure heterospecific samples – 1). OY: goodness of correspondence between assessment of predictive values by equations and regression models.

Therefore, in order to predict *Ptros* using "*Ptros* by *PT* calculator" one should use the most dissimilar samples to assess *P(T|edu)* and *P(T|tros)* as calculator parameters. In order to predict *P(edu|E)* and *P(tros|T)* using "genotype by morphotype calculator" one should assess the parameters using the most mixed samples. However, the *Ptros* as input in Eq.1-2 should be calculated as in the previous case i.e. using the most dissimilar samples and Eq. 3. To illustrate the approach (see **Fig 3**) for *WSBL*, *BH*, *GOM* and *BALT* we used pooled sets of samples with *Ptros* <0.1 and >0.8 to calibrate "*Ptros* by *PT* calculator" and 0.45 < *Ptros* < 0.65 to calibrate "genotype by morphotype calculator" as described above (samples included are indicated in **S1 Table**). We used pooled but not individual samples to avoid basements due to small sample size. The given ranges of *Ptros* were used because of the lack of *M. trossulus*-dominated samples in most sets. For *NORW* and *SCOT* we pooled all the samples because of the lack of data.

Visual inspection of **Fig 3a** revealed a nearly ideal correspondence between regression lines and predictions of "*Ptros* by *PT* calculator" in the case of *WSBS* and *GOM*. In the case of *SCOT*, where only two samples were available, the line derived from Eq3 approached the Y=X line. A rather close though not ideal correspondence was observed in the case of *BALT*, deviation being due to a very high slope term of the regression. *Ptros* was slightly underestimated by the calculator in this case. The worst correspondence between Eq3 and Model 4 was observed in the case of *NORW* and *BH*. In *BH Ptros* was severely overestimated by the calculator, which was opposite to the situation in *BALT*. In *NORW* both regression and predictions of calculator were severely affected by the outlier sample.

As for the "genotype by morphotype calculator", the predictions generally were in good correspondence with the regression lines (calculator's lines were within 95% CI of regressions). Deviations were observed for *P(tros|T)* predictions in *WSBL* for *Ptros*<0.25 and *P(edu|E)* in *GOM* for *Ptros*>0.6 i.e. in the *Ptros* ranges corresponding to a small prevalence of the species.

An exercise with the "lazy *Ptros* by *PT* calculator", in which the highest and the lowest *PT* in samples from regional sets are used as *P(T|tros)* and *P(T|edu)* parameters of Eq. 3, had the following results (**S2 Fig**). In *WS*, *BL* and *GOM* correspondence between the observed and the predicted *Ptros* values in samples was generally good. In *BH*, *Ptros* was slightly overestimated by the calculator due to the absence of pure *M. trossulus* samples in the data and the formal choice of a numerically small (N=22; see **S1 table**) sample with the highest *PT* but not the highest *Ptros* as the "calibrating" one. For *BALT* and *NORW* discrepancies were much stronger, the reasons being the same as in case of "*Ptros* by *PT* calculator" (see above).

S2 Fig. Correspondence between empirical estimates of *Ptros* in samples and predictions of the "lazy *Ptros* by *PT* calculator" (the highest and the lowest *PT* in samples from regional sets are used as *P(T|tros)* and *P(T|edu)* parameters of Eq. 3). Dots – estimates, solid line – linear regression, dashed line – Y=X line.

Discussion

The knowledge about the taxonomic structure of populations and a rough classification of individuals into "species" is often more valuable to the blue mussel researchers than the precise information about the genetic affinity (e.g. Structure q-value) of any given mussel. In the light of this, our finding that *M. edulis* and *M. trossulus* genotypes in the White Sea differed by the frequencies of the shell morphotypes [16] seemed very promising. It gave hope that this knowledge could be obtained for these species by a quick examination of the inner side of the shells, without genotyping, which is expensive, time-consuming and requires soft tissues (genotyping of shell material is possible [42,43] but is not yet routine practice). In this study we reanalyzed abundant data from [16] and derived robust relationships between the proportions of the morphotypes in the populations and their taxonomic structure as well as

between the proportions of the morphotypes in populations and the probabilities of mussels of different morphotypes being *M. trossulus* and *M. edulis*. These relationships could be used for a reliable prediction of the taxonomic structure of any population in the White Sea. Moreover, any mussel in an equally mixed population could be identified as *M. trossulus* or *M. edulis* with the accuracy of about 80% (a bit less than it was predicted basing on frequencies of the morphotypes in pooled data on the White Sea *M. edulis* and *M. trossulus*, see Introduction). With the increasing imbalance between the species (and hence the morphotypes) in a population, the identification of the dominant species became more reliable though the identification of the minor species became less so.

The ultimate goal of our study was to find out whether the possibility of identifying *M. edulis* and *M. trossulus* by the morphotype was a "privilege" of the researchers working at the White Sea or whether this approach could be used for identification of these two species worldwide. Though our data on the contact zones between the species outside northern Russia were limited, our results indicate that this approach may be useful everywhere since interspecific differences in the morphotype frequencies were ubiquitous and unidirectional. However, its utility is evidently different for different contact zones.

Though the hypotheses that different mussel species may differ by the extent of the nacreous layer development under the ligament nympha was already suggested by Zolotarev, Shurova [44] and Vervoenen et al. [45], the morphotypes were actually applied to species identification by Khaitov et al. [22] (see below). Here we show that the morphotype test is a promising tool. Once it has been evaluated, i.e. associations between morphotypes and species-specific genotypes have been worked out at the individual and the population levels, it will hopefully deserve more attention from the blue mussel researchers.

To note, another method for a fast morphological diagnosis of *M. trossulus* and *M. edulis* was suggested by Beaumont et al. [19], who showed that commercially damaging "fragile mussels" in Scottish *M. edulis* plantations were genetically similar to *M. trossulus*. The fragile mussels differed from *M. edulis* (and the reference *M. galloprovincialis*) by a combination of shell traits including shape, the degree of expression of growth ridges and the color of the inside. The promising identification method was however not developed further. A comparison of the photographs of shells in Beaumont et al. (2008) with our Barents Sea samples (**S1 Fig E**) shows that the interspecies differences in the Barents Sea are less striking than in Scotland.

We will start with the discussion of the patterns of variation of the morphotype frequencies revealed in our study. Then we will discuss the applicability of the morphotype test in different contact zones. In the closing section, the limitations of single-marker taxonomic tests for blue mussels and other taxa will be outlined.

Variation of morphotype frequencies among conspecific populations

Some variation in the morphotype frequencies was observed among putatively pure conspecific populations sampled at a distance from the contact zones. Samples of pure *M. edulis* from the temperate seas (i.e. all except those from the eastern Barents Sea and Greenland) were nearly monomorphic for the E-morphotype, while the northern samples were more polymorphic and diverse. In turn, the reference populations of *M. trossulus* from the northwestern and northeastern Pacific (Washington) were nearly monomorphic for the T-morphotype. Nevertheless we cannot necessarily exclude the possibility of geographic variation in *M. trossulus* in its ancestral Pacific range or confirm that the T-morphotype is the "ancestral" state for this species. Zolotarev [46] identified morphotypes in small samples of genotyped mussels (from [7]) and found elevated frequencies of the E-morphotype in

653 *M. trossulus* from Oregon (northeastern Pacific). Those data should be treated with caution, however,
654 because he used another classification of the morphotypes and identified them macroscopically, and
655 also because Oregon is close to a contact zone between *M. trossulus* and *M. galloprovincialis* [7]; the
656 latter species is characterized by the E-morphotype [44,46].

657 In *M. trossulus* the morphotype frequencies varied between the contact zones, and elevated frequencies
658 of E-morphotypes were found in Norway and, especially, in the Baltic Sea. The variation within
659 contact zones was mostly due to the few "outlier" samples from the Gulf of Maine and Norway. On the
660 contrary, *M. edulis* showed little variation between zones, the T-morphotype being universally rare. In
661 a notable exception the T-morphotype frequency was clearly elevated (up to 40%) in samples from
662 saline localities (> 30 ppt) in Kola Bay and surroundings. Similar salinity-related variation was found
663 in *M. edulis* from the more eastern areas of the Barents Sea, at some distance from the contact zone
664 between these species along the Kola Peninsula coast.

665 Finally, an analysis of the abundant material from the White and the Barents Sea demonstrated how the
666 morphotype frequencies varied with the taxonomic composition of the mixed populations. The
667 frequencies of the T-morphotype increased both among *M. edulis* and among *M. trossulus* genotypes
668 with the increasing prevalence of *M. trossulus* in the samples.

669 *Unusual features of M. trossulus from Norway and the Baltic Sea*

670 *M. trossulus* from the Baltic Sea and Norway were characterized by extremely high frequencies of the
671 E-morphotype. Two hypotheses, which are not mutually exclusive, can be offered to explain this
672 phenomenon. One hypothesis likens the morphotypes or, more specifically, the underlying hypothetical
673 genes, to alleles of taxonomically diagnostic loci that can introgress between species as a result of
674 extensive hybridization and backcrossing. Genetic studies show that the Baltic *M. trossulus* hybridizes

675 more freely with *M. edulis* and is stronger introgressed by *M. edulis* genes than any other Atlantic
676 population [3,47]. Due to its mixed genetic nature, the Baltic mussel is sometimes considered as a
677 unique *M. edulis* x *M. trossulus* hybrid swarm, which is fundamentally different from the "oceanic" *M.*
678 *trossulus* [3]. While the genetic data from Norway are limited, hybridization is apparently more
679 extensive there than in most other contact zones though not as extensive as in the Baltic [3,5]. Besides,
680 it is evident that the local Norwegian *M. trossulus* populations can be strongly introgressed by *M.*
681 *edulis* genes [48].

682 In the second hypothesis, the frequency of the T-morphotype in *M. trossulus* is reduced under the
683 influence of some environmental factors, both micro- and macrogeographical. We suspect that the
684 nearly zero frequencies of the T-morphotype in the "outlier" samples (one from Norway, almost from
685 the same place as the other Bergen samples, and two from Cobscook Bay in the Gulf of Maine [CBCP,
686 CBSC in **S1 Table**]) could be explained by the impact of some cryptic local factors, though a more
687 prosaic explanation such as the mislabeling of mussels in the collections cannot be entirely ruled out.

688 *Salinity-related variation in M. edulis*

689 While local factors putatively affecting morphotype frequencies in *M. trossulus* remained cryptic, in
690 the Barents Sea we managed to identify one such factor governing morphotype frequencies in *M.*
691 *edulis*: salinity or a factor/factors linked to salinity. The eastern part of the Barents Sea, where this
692 variation was evident, is also the coldest. The border between the more temperate populations of *M.*
693 *edulis* with "normal" (high) frequencies of the E-morphotype and the more Arctic populations with
694 lower frequencies of the E-morphotype in oceanic habitats runs somewhere between North Cape and
695 the Kola Bay (Fig 1). This area has mean annual, summer and winter sea surface temperatures of about
696 6°C, 9°C and 4°C, respectively (*http://esimo.oceanography.ru/*).

697 The functional significance of the morphological character underlying the E- and the T- morphotype
698 (the presence/absence of the nacreous layer under the ligament) is unclear. However, we suspect that
699 the morphotypes might differ in conspecifics by the degree of development of the nacreous layer itself
700 and thus in the thickness and strength of the shell. The nacreous shell layer is mechanically the
701 strongest [49]. *M. trossulus*, which is usually marked by the T-morphotype, generally has a thinner
702 nacreous layer and a more fragile shell than *M. edulis* (cf. [19], see above). *M. edulis* of the T-
703 morphotype might have an underdeveloped nacreous layer and a thinner shell than the conspecifics of
704 the E-morphotype.

705 Can we expect the shell thickness and structure to differ in mussels from saline (oceanic) and brackish
706 (estuarine) environments in the Arctic? Apart from the low temperatures, the Arctic Sea is
707 characterized by a reduced concentration of calcium carbonates [50] and, seasonally, by low
708 concentrations of planktonic algae, which the mussels feed on [51]. Estuarine habitats are generally
709 characterized by the lowest saturation of carbonates but the highest concentrations of food (seston),
710 which is due to the riverine discharge [52]. This is exemplified by the highest biomasses of *Mytilus* in
711 estuaries in the Barents Sea [53] and elsewhere [12]. Mussels need both calcium carbonates and energy
712 for shell growth and maintenance. In estuaries, the nacreous layer of the mussel shell is prone to
713 dissolution and corrosion [54] but the mussels can still keep their shells strong if the food is sufficient
714 [52,54]. If the food is limited, the energy is likely to be allocated to the maintenance of the somatic
715 mass rather than the conservation of the shell ([54] and references therein).

716 Our hypothesis explaining the assumed differences in the degree of the nacreous layer development
717 between *M. edulis* from the brackish and the saline localities in the Arctic is that in the estuaries the
718 mussels allocate more energy for shell maintenance thus keeping their nacreous layer thick while in
719 less corrosive but more famished oceanic habitats they allocate more energy for somatic growth

keeping their nacreous layer thin. As a result, the majority of *M. edulis* from the saline localities has the undeveloped nacreous layer. It is noteworthy that in the areas where *M. edulis* demonstrated salinity-related variation, the morphotype frequencies in *M. trossulus* varied negligibly. This could be attributed to a generally lower shell plasticity in "oceanic" (non-Baltic) *M. trossulus* than in *M. edulis* in response to the environmental stressors ([55], see [22] for more discussion).

Noteworthy, reduced frequencies of the E-morphotype were revealed not only in the eastern Barents Sea but also in northernmost populations of *M. edulis* from Greenland and the Gulf of Saint Lawrence in western Atlantic (**Fig 1**). This indicates that this is an Arctic phenomenon. Unfortunately, the salinity in the sampling localities in the latter two areas is unknown.

Variation with the taxonomic structure

A positive correlation of the T-morphotype frequencies both in *M. edulis* and *M. trossulus* with the prevalence of *M. trossulus* in the representative data sets from the White and the Barents Sea was to be expected, bearing in mind that *M. edulis* and *M. trossulus* genotypes are defined by the dominance of the conspecific genes in multilocus genotypes. Hence both genotypes included purebreds as well as hybrids. From a detailed analysis of the White Sea data [16] we know that the frequencies of hybrids are approximately the same in all the samples (18% on the average), hybrids are intermediate in morphotype frequencies between purebred *M. edulis* and *M. trossulus* but usually closer to species dominating the population [16]. This means that in our analyses "*M. edulis* genotypes" from *M. trossulus*-dominated populations included mostly hybrids with an increased frequency of the T-morphotype as compared to the "*M. edulis* genotypes" in *M. edulis*-dominated populations. In turn, "*M. trossulus* genotypes" from *M. edulis*-dominated populations included mostly hybrids with a decreased frequency of the T-morphotype as compared to such genotypes in *M. trossulus*-dominated populations.

This is the cause of the observed unidirectional variation in the morphotype frequencies among *M. edulis* and *M. trossulus* genotypes with the changing taxonomic structure of populations. To note, the variation of sensitivity and specificity of clinical diagnostic tests with the changing disease prevalence is often observed [56]. For instance, a patient population with a higher disease prevalence may include more severely diseased patients, and the test would consequently perform better [56].

Applications of the mussel morphotype test

The morphotype test can be universally applied as an alternative to genotyping in three fields. Firstly, it can be used for monitoring the species composition of commercial and wild populations, in particular those used in the "mussel watch" contaminant monitoring programs, because deviations of the morphotype frequencies may be a warning sign of the taxonomic change. Secondly, it may prove useful for mapping the species distributions. Detailed mapping is likely to require a great number of samples because the distribution of the species in contact zones is usually highly mosaic (see [16] and references therein). Thirdly, the morphotype test can be used when only dead mussel shells are available, e.g. for interpretations of the taxonomic structure of natural history collections or samples of dead shells left behind by some mussel predators.

Identification of taxonomic structure of populations from contact zones

A reliable application of the morphotype test requires good genotyped references. Ideally, empirical relationships should be established between the morphotype frequencies and the taxonomic structure of populations in a given contact zone, as was done in our study (**Table 2**). Even our regressions require further refinement for all the contact zones except northern Russia, since they are based on a relatively small number of samples. On a reassuring note, for mixed populations from the Baltic and the Gulf of Maine as well as for the populations from northwestern Greenland and the North American coast north

764 of the Gulf of Maine unexamined in this study, collections of genotyped mussels probably remain from
765 previous extensive population genetic studies (e.g. [5,7,57–59]). The collections could be used for
766 further calibration of the morphotype test for these contact zones. If such an effort is undertaken for
767 Greenland and high latitude American populations, salinity and trophic conditions should be
768 considered as a potential covariates of the morphotype variation.

769 The relationships between the morphotype frequencies and the taxonomic structure of populations will
770 have to be established *de novo* in understudied or, potentially, new contact zones. Should the
771 genotyping of more than a few samples covering the range of the morphotype frequencies prove
772 impractical, the relationships could be approximated using the data on at least two genotyped samples
773 with the most contrasting structure (ideally, pure *M. edulis* and pure *M. trossulus*) and the "*Ptros* by *PT*
774 calculator" (Eq. 3) (cf. **Fig 2**). At the very least, the relationships could be weighed roughly without
775 any genotyping, by taking the minimal and the maximal morphotype frequencies in regional
776 populations as hypothetical corresponding frequencies in pure *M. edulis* and pure *M. trossulus*
777 populations ("the lazy *Ptros* by *PT* calculator", cf. **S3 Fig**). Naturally, such predictions should be
778 treated with the greatest caution.

779 We claim that the morphotype test may be useful for the detection of new contact zones and for their
780 formal genetic description. The procedure would involve a preliminary selection, with the help of the
781 morphotype frequencies, of the purest samples needed for the verification of the species identity and of
782 most mixed ones needed for the assessment of the extent of hybridization and mixing.

783 In case of historical or archaeological collections, the only way to translate the proportion of the T-
784 morphotypes in the samples into the taxonomic structure is to resort to the actualistic principle. If the
785 correspondence between the morphotypes and the genotypes was assessed in the area of the sample

786 origin, one can use this information for retrognosis. This should be possible for quantitatively
787 representative samples though not for small samples or single shells. Unfortunately, the morphotype
788 test is unlikely to be useful for the interpretation of paleontological data since the morphotype
789 frequencies in conspecifics are affected both by geography and by the local oceanographic conditions,
790 which are variable at a large time scale.

791 *Individual identification*

792 The possibility to identify individual mussels by the morphotype seems to be the "privilege" of
793 researchers working at the White Sea and brackish environments of the Barents Sea. The morphotype
794 test also seems to be promising for individual assignment in the Gulf of Maine, except in the outlier
795 samples (see above) and, possibly, in Scotland (unfortunately, the Scottish populations were
796 represented in our analysis only by two samples). In the Baltic Sea and Norway the morphotype test
797 worked reliably only for *M. trossulus* mussels, while in the saline areas in the Barents Sea it did so only
798 for *M. edulis* mussels.

799 The morphotype test was already used for practical assignment of individuals in a study of prey
800 selection of the predator starfish *Asterias rubens*, feeding on mixed mussel populations in the White
801 Sea, [22]. We sampled mussels in populations with high and low T-morphotype frequencis, and mixed
802 them in equal proportions in an experimental setup. The predators selectively consumed mussels of the
803 T-morphotype, which was interpreted as a preference towards *M. trossulus* [22]. Now we know that an
804 alternative and probably more formal experimental design could be to use sympatric mussels of T- and
805 E-morphotypes from the most mixed population. Under both designs, the accuracy of individual
806 assignment of experimental mussels would be nearly the same.

We would like to stress that, if one plans to use the morphotype test for individual assignment, reliable genetic references are indispensable. These could be either empirical relationships between the proportions of the morphotypes in the samples and the probabilities of mussels of different morphotypes being *M. trossulus* or *M. edulis* or control genotyping of mussels from the populations of interest. Still, it is noteworthy that the accuracy of individual identification of mussels could be approximated basing on the morphotype frequencies in three "calibration" samples (those with the maximum, the minimum and the intermediate proportions of species) and eq. 1-3 (cf. **Fig 3**).

Pitfalls of the morphotype test

The morphotype test comes with pitfalls. One of the evident risks is an underestimation of *M. trossulus* by morphotypes in some populations, such as those in Norway and the Gulf of Maine, which were the sources of the "outlier" samples. Another is the bias generated by a non-random association of morphotypes with size (or age) of conspecific mussels such as was observed in very rare (about 2%) samples. A further risk are uncertainties in the application of the test to populations from intermediate salinities (about 30 ppt) in the Barents Sea. To note, four out of the five samples with a significant non-random association between the morphotypes and the size were from Tyuva inlet (**S4 table**) right at the border between brackish and full saline areas of the Kola Bay. It is possible that temporal or ontogenetic trends in the morphotype frequencies are a local Barents Sea phenomenon related to the unusual salinity conditions as in Tyuva.

Uses and abuses of single marker taxonomic tests

Traditional species identification relies on diagnostic (fixed) morphologic traits of the organism, usually included in the diagnosis. In the terms of the probability theory, it means that the probability of an individual with a species-specific diagnostic marker being a representative of the species in question

829 is equal to one: *P(species|trait)* = 1. However, in practice the probability can be lower for two reasons.
830 First, because of scoring errors related to the researcher's skills or the defective condition of the
831 specimen. Second, for the ambiguity in the diagnosticity of a trait. It is generally impossible to
832 determine whether diagnostic characters are indeed fixed if the sample size is finite [60]. Hence, in
833 practice, for diagnostic markers $P(species|trait) \leq 1$.

834 Some taxa, however, lack diagnostic characters and have to be identified on the basis of semi-
835 diagnostic ones. This is the case with the blue mussels [7]. In case of semi-diagnostic traits, the
836 researchers do not identify the species of a given individual but assess the probability of its assignment
837 to one or another species. For these traits, $P(species|trait) < 1$. Similarly, dealing with population
838 assessment we assess the probabilities of finding the representatives of one or another species in a
839 sample but not the true proportion. The most critical point is that *P(species|trait)* is not constant but
840 varies, yet in predictable manner, with the prevalence of a species in a range [0;1].

841 A correct application of tests based on semi-diagnostic markers, such as clinical diagnostic tests,
842 ultimately requires a "reference standard" used for verification of the index test results [24]. In our case
843 study of the blue mussels, we used as references the groups of multilocus genotypes (from 4 to 171 645
844 loci depending on the geographical sample set) defined by the dominance of alleles characteristic of
845 one or the other species. These groups did not represent true species. They included hybrids, some of
846 which (e.g. first- and second generation hybrids) were assigned into groups randomly. To note,
847 multilocus genotyping is seldom employed for identification of cryptic mussel species. Most studies
848 rely on singular or few "standard" diagnostic PCR-based markers, usually nuclear Me15/16 and ITS
849 and mitochondrial COI or 16S markers [11]. Offering the morphotype test as a rough but cost-efficient
850 alternative to genotyping, we have to assess its reliability as compared to single- and few locus tests. It
851 has been long known that the efficiency of "diagnostic" markers for discrimination between *M. edulis*

852 and *M. trossulus* is different in contact zones in western Atlantic (i.e. the Gulf of Maine) and the Baltic
853 Sea [1]. In the Northwest Atlantic the species are nearly fixed for alternative alleles at Me15/16, ITS
854 and mitochondrial markers, while in the Baltic Sea intraspecific differences at these loci are 20%, 32%
855 and 0%, respectively, due to a introgression of *M. edulis* genes into the local *M. trossulus* genome [1].
856 For comparison, the differences in morphotype frequencies between species in the Gulf of Maine and
857 the Baltic Sea are 44% and 6%. As far as we know, the efficiency of taxonomic tests based on singular
858 or a few "standard" loci has not been carefully evaluated for other *M. edulis – M. trossulus* contact
859 zones, though some attempts have been made (see [3,61]). The recent population genomic studies of
860 hybridizing *Mytilus* species indicate that these species can altogether lack fixed genetic differences due
861 to ubiquitous introgression and that loci can introgress in unpredictable manner in different contact
862 zones [47,62]. On these grounds, the conventional approach to mussel species identification based on
863 singular molecular markers has been criticized [62]. We do not claim that the morphotype test would
864 fare better than most single-locus taxonomic tests in any contact zone between *M. edulis* and *M.*
865 *trossulus*. At the same time, we would like to point out that the performance of these genetic tests has
866 not been evaluated in most contact zones, while that of the morphotype test has been.

867 A situation when one has to rely on a singular "informal" semi-diagnostic character to distinguish
868 morphologically such old evolutionary lineages as *M. edulis* and *M. trossulus* is certainly uncommon in
869 taxonomy. At the same time, it is not unique. There are other taxa, which lack fixed diagnostic
870 morphological characters and are identified by semi-diagnostic traits, individual or complex such as
871 like the coordinates of multifactorial analysis. These taxa include subspecies defined according to the
872 75% rule [63], cryptic species with statistical differentiation [64] and hybridizing species that
873 secondarily lost fixed differences due to introgressive hybridization [65]. Therefore, we hope that our
874 experience of dealing with a non-fixed taxonomic character would be interesting not only to our

colleagues working with blue mussels but also to the researchers who study sympatric taxa with vague morphologies and semi-isolated gene pools.

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Dear Editor, please find enclosed our manuscript “Species identification based on a semi-diagnostic marker: evaluation of a simple conchological test for distinguishing blue mussels Mytilus edulis L. and M. trossulus Gould” by Khaitov et al. Which we would like to submit for publication as a research article in Plos One. We hope you will find is suitable for publication.

Common blue mussels *Mytilus edulis* and *M. trossulus* lack reliable taxonomic morphological traits and are generally identified with the help of multilocus genotyping. Any cues for distinguishing them without genotyping would save much research effort. We have previously shown (Katolikova et al. 2016 in PLoS One) that a simple conchological trait is asymmetrically distributed across this two taxa in the White Sea and can be considered as a semi-diagnostic taxonomic marker. In the present research article we assess the applicability of this trait to taxa identification in sympatric and allopatric populations of *M. edulis* and *M. trossulus* all over the world. We found that the trait usually, though not always, had a high predictive power. Since it is not diagnostic but semi-diagnostic, we applied the approaches developed in evidence-based medicine where identification of a syndrome is associated with probabilistically distributed diagnostic test outcomes. This approach may apply equally well to other taxa identified by semi-diagnostic traits.

The results of the work have not been submitted anywhere. We use some materials published previously in PLoS (Katolikova et al. 2016) but we considered them as a part of broader material.

We suggest Dr. Donald James Colgan (Australian Museum, AUSTRALIA) as an Academic Editor to handle our manuscript.

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Figure 1

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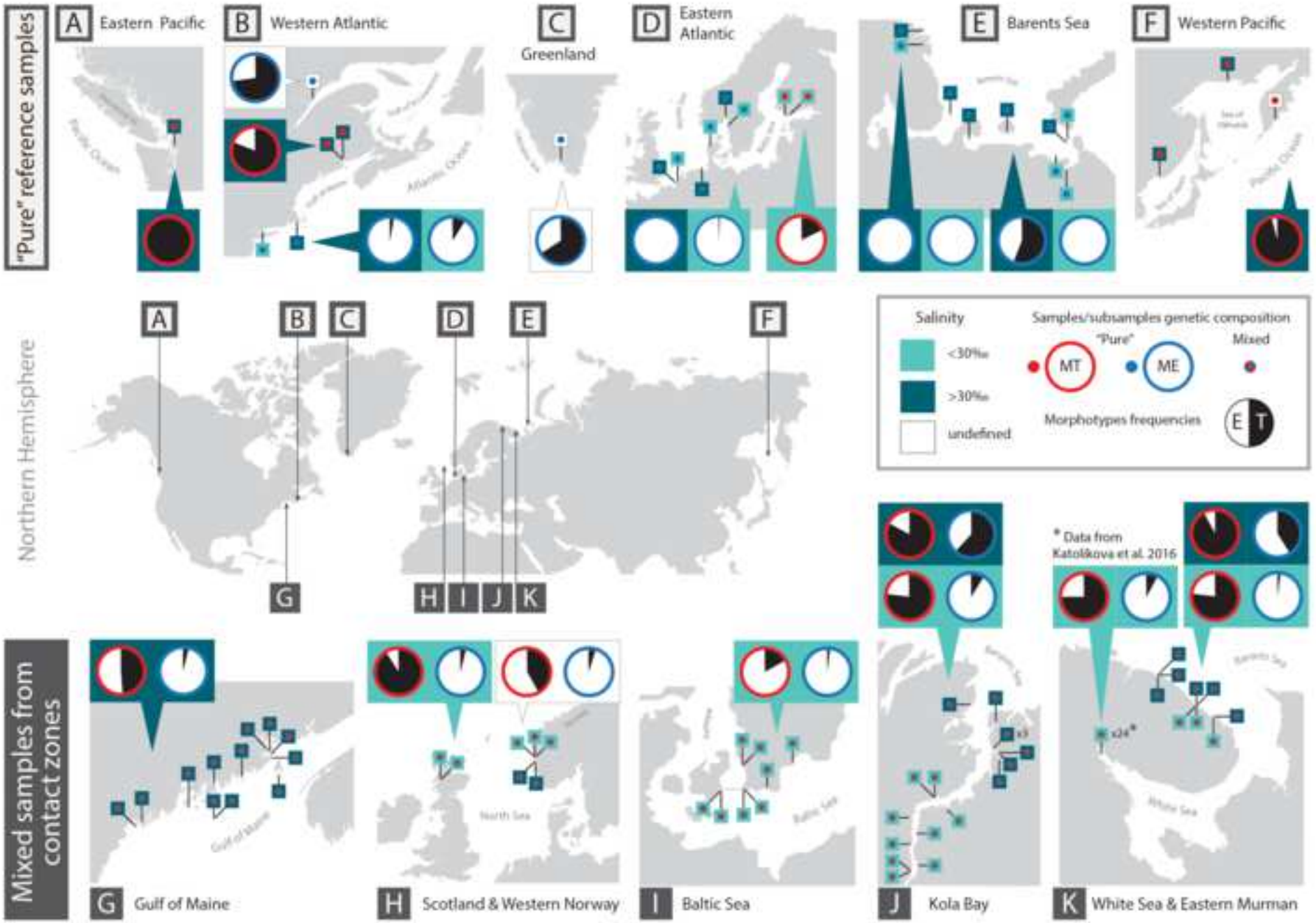

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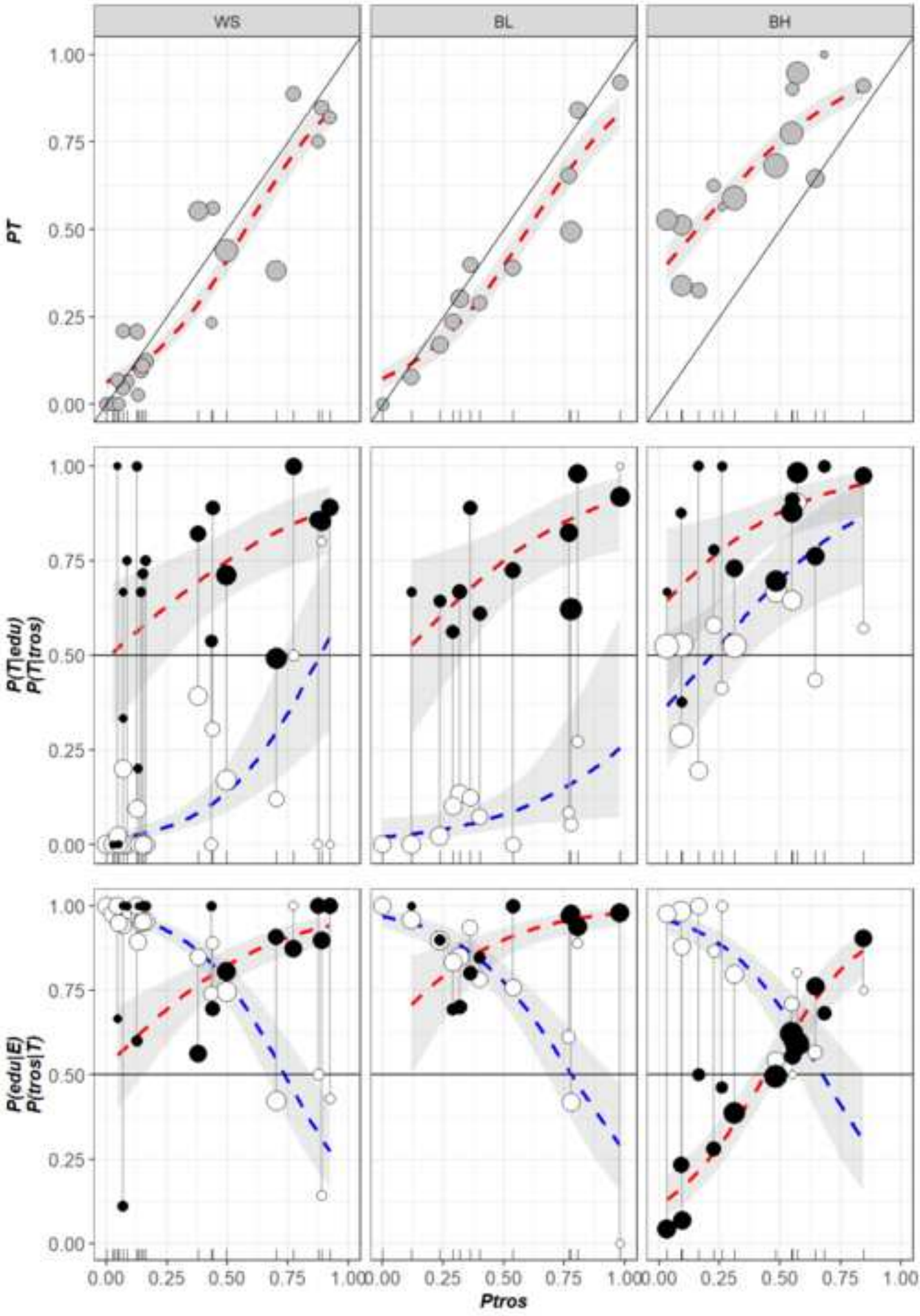

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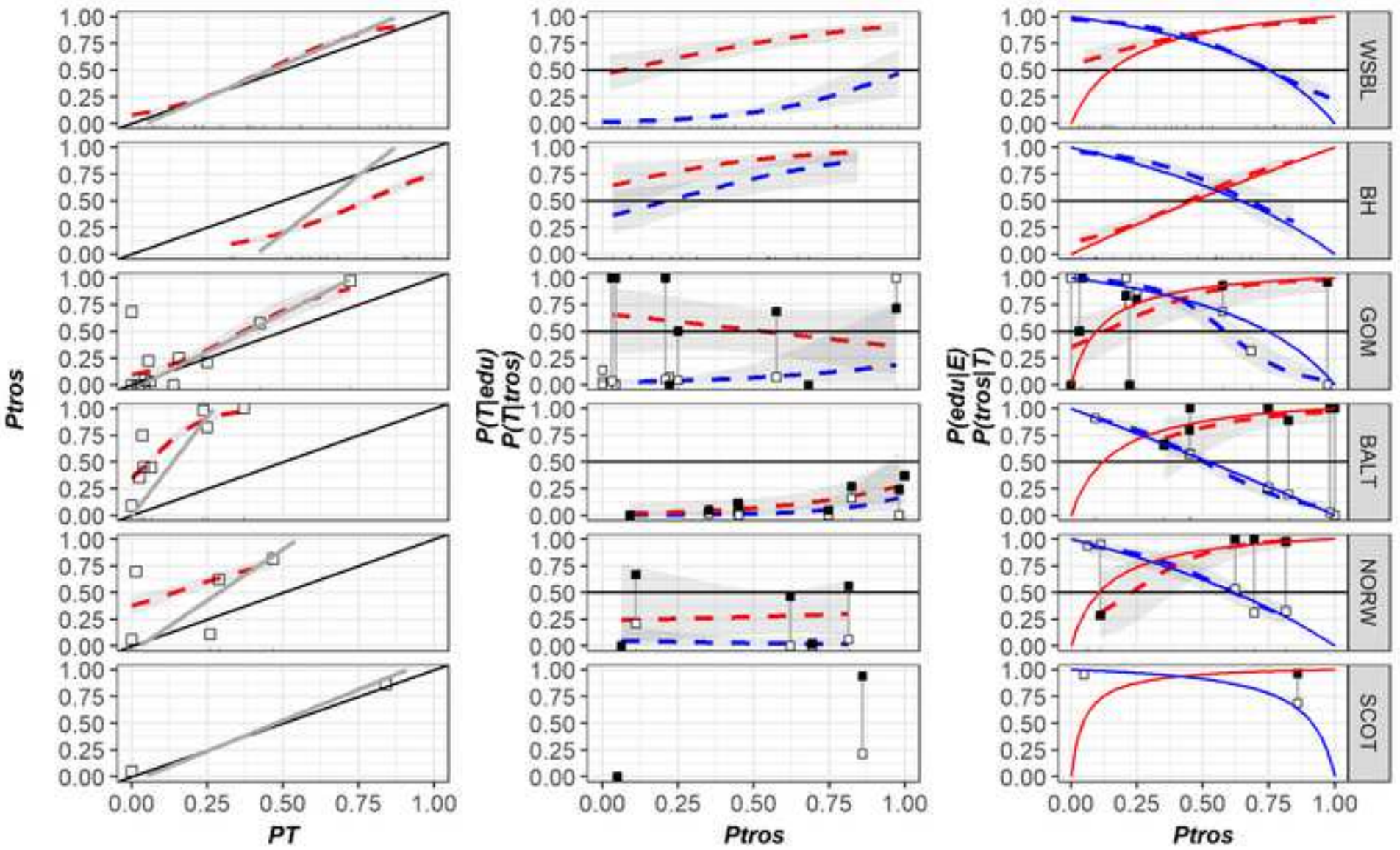

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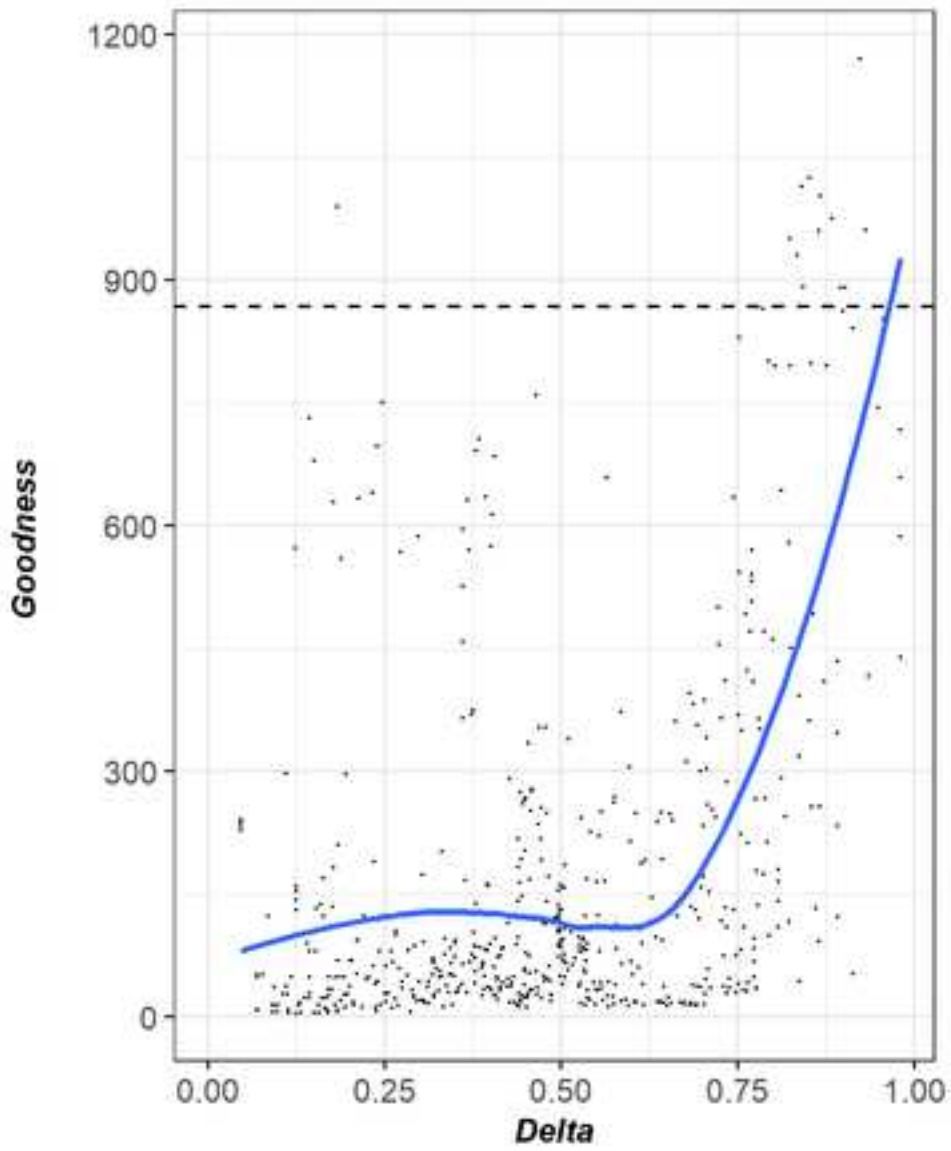

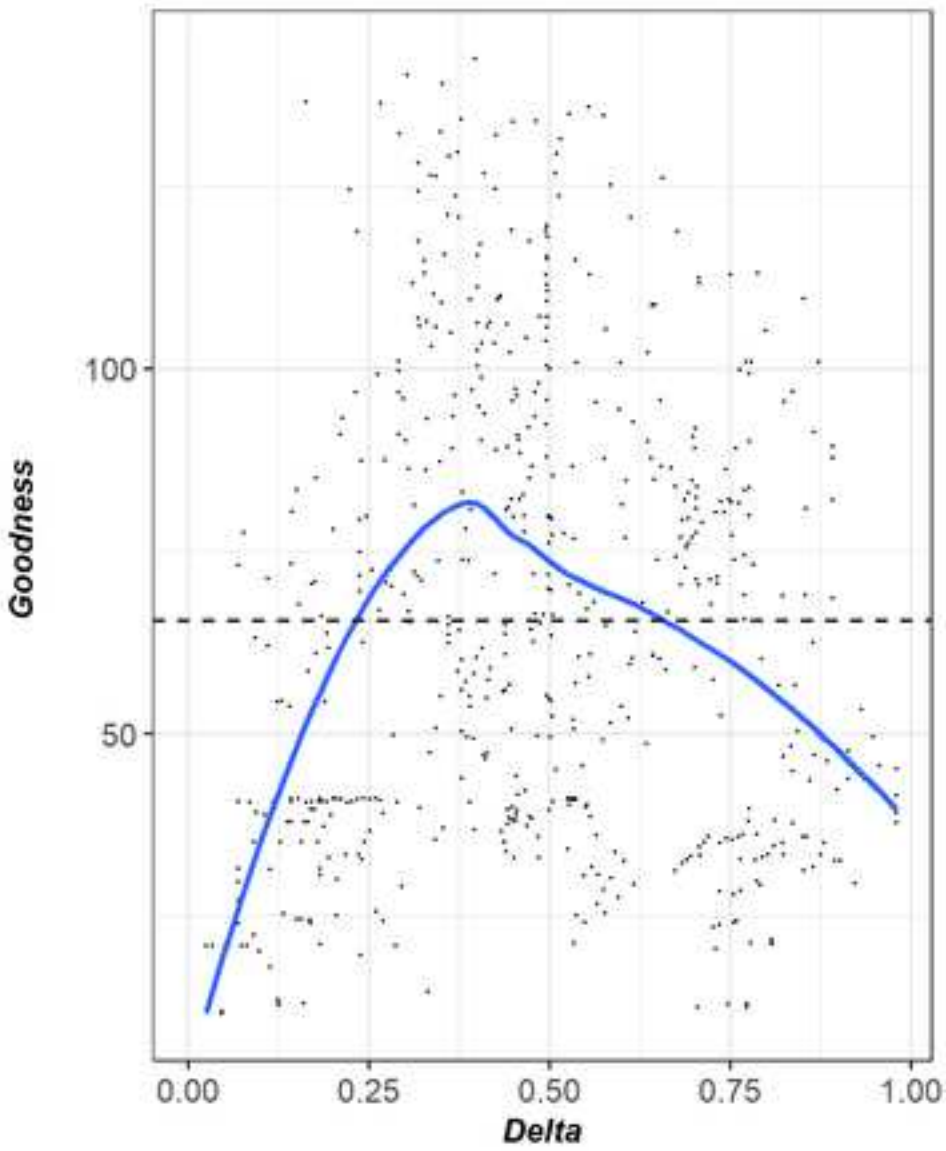

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