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ORIGINAL RESEARCH

Mapping Loci for Fox Domestication: Deconstruction/Reconstruction of a Behavioral Phenotype

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Abstract During the second part of the twentieth century, Belyaev selected tame and aggressive foxes (*Vulpes vulpes*), in an effort known as the "farm-fox experiment", to recapitulate the process of animal domestication. Using these tame and aggressive foxes as founders of segregant backcross and intercross populations we have employed interval mapping to identify a locus for tame behavior on fox chromosome *VVU12*. This locus is orthologous to, and therefore validates, a genomic region recently implicated in canine domestication. The tame versus aggressive behavioral phenotype was characterized as the first principal component (PC) of a PC matrix made up of many distinct behavioral traits (e.g. wags tail; comes to the front of the cage; allows head to be touched; holds observer's hand with its mouth; etc.). Mean values of this PC for F1, backcross and intercross populations defined a linear gradient of heritable behavior ranging from tame to aggressive. The second PC did not follow such a gradient, but also mapped to VVU12, and distinguished between active and passive behaviors. These data suggest that (1) there are at least two VVU12 loci associated with behavior; (2) expression of these loci is dependent on interactions with other parts of the genome (the genome context) and therefore varies from one crossbred population to another depending on the individual parents that participated in the cross.

Keywords Behavior genetics · Domestication · Social behavior · *Vulpes vulpes* · *Canis familiaris*

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Introduction

Behavior is a complex phenotype that involves the interaction of organisms with their physical and social environments. It can vary from the well-defined reaction of plants' stomates when exposed to increases in temperature and radiation, to behaviors that depend on communication between species such as predator–prey relations, or domestication that involves changes in the behavioral relationship between animals and man. The latter more complex phenotypes are gestalts comprised of many smaller traits, that are often overwhelmed by single major responses such as the fight or flight response that frequently occurs when individuals confront each other. When such confrontations arise, a range of responses may result depending on the way an animal assesses the changing situation, and its ability to "read" the intent of the other individual or to signal its own intentions. Within a species or subspecies, these signals may

be well developed and understood; between species such communication is more difficult, requiring the dissection of the gestalt into its component parts. It now seems clear that domestication of the dog involved changes that allowed the wolf-changed-to-dog to read human intent and based on that analysis to develop a gestalt involving a reserved trust or even an invitation to entertain a physical relationship (Hare et al. 2002; Gácsi et al. 2004, 2005; Topál et al. 2005; Miklósi 2009). The ability to communicate with humans in a positive way and read human cues has been documented by Hare et al. (2005) in the domesticated strain of foxes described below and has been observed in wolves raised from a very early age by humans (Udell et al. 2008; Gácsi et al. 2009).

In the latter half of the twentieth century Belyaev and Trut carried out an experiment (Farm-fox experiment, Trut 1999, 2001; Trut et al. 2009) to recreate the evolution of canine domestication by selecting foxes (*Vulpes vulpes*) that were either more tame or more aggressive than unselected foxes. Within a few generations they developed two distinct populations of silver fox (a coat color variant of the red fox) whose behavioral repertoires, when interacting with humans, differed remarkably. These populations exhibited differences in many distinct and specific behavioral traits: their position within their cage when a human approached; the noises that they made, the position of their ears and tail; and much more obvious traits such as the willingness and desire to be touched as opposed to their eagerness to attack and bite.

Noting that these gestalts were manifested by multiple traits, Kukekova et al. (2008) developed a videotaped analysis of behavior comprised of multiple specific observations of physical reactions to the approach of an investigator to the caged fox and its further reactions to the investigator trying to open the cage and touch the animal. In this manner, they succeeded in developing an objective measure of tame versus aggressive behavior out of the somewhat more subjective and qualitative test that had been used in the selection of the tame and aggressive populations (Trut 1999, 2001; Kukekova et al. 2005; Trut et al. 2009). Ultimately this video test consisted of evaluating each fox in a binary manner (yes/no) for a set of traits that involved the fox's body language, actions and position with respect to investigator. Using principal component (PC) analysis it was possible to correlate variation in such reactions into quantitative measures of behavioral phenotypes. The selection of the two founder populations had already established that this behavioral axis, tame versus aggressive, was heritable (Trut 1980) and the motivation behind quantifying the phenotype was to identify genetic loci that controlled the behavioral change.

Recent results from dog/wolf genome comparisons have suggested particular loci as involved with domestication (vonHoldt et al. 2010). Thus, identification of loci for domestication in the fox, an out-group among modern canids, could provide independent validation of the wolf/dog results. Here we report such validation and describe a region of the fox genome that regulates segregation of two behaviors: traits that give rise to the gestalt of domestication (i.e. differences between tame and aggressive animals); and traits characterized by active versus passive responses to humans.

Materials and methods

Animals, pedigrees, and samples

We studied six fox populations that are maintained at the experimental farm of the Institute of Cytology and Genetics of the Russian Academy of Sciences in Novosibirsk, Russia. These populations included the original tame and aggressive fox strains and experimental crosses that were produced in this study: F1—intercross between the tame and aggressive strains; BCT—backcross to the tame strain, BCA—backcross to the aggressive strain, and F2.

Two sets of backcross-to-tame pedigrees (BCT_1 and BCT_2) were developed by crossing F1 foxes to the tame strain. BCT_1 (163 offspring in informative generation) and BCT_2 (130 offspring) were produced in two consecutive years. One set of BCA pedigrees (202 offspring) was produced by crossing F1 foxes to the aggressive strain. Two sets of F2 pedigrees (F2_1 and F2_2) were produced by breeding F1 foxes to each other. F2_1 (90 offspring) and F2_2 (160 offspring) were developed in two consecutive years. All F1 and BC pedigrees were produced in reciprocal manner with respect to parental gender and population of origin.

Different F1 parents were used to produce the backcross-to-tame and F2 pedigrees. Two backcross-to-tame (BCT_1 and BCT_2) populations were produced using mostly the same F1 parents but mostly different tame parents, only 20% of the tame parents were common to both populations. About 60% of F1 parents were common to both backcross-to-tame and backcross-to-aggressive populations.

Blood samples were collected and DNA was extracted using Qiagen Maxi Blood kits (Qiagen, Valencia, CA) or using phenol–chloroform extraction methods (Gilbert and Vance 1994).

Assignment of fox behavioral phenotypes

Fox behavior was tested in the standard test as described in detail in Kukekova et al. 2008. Each fox was tested at 5.5–6 months of age at least twice and each test was videotaped. The test was designed to evaluate fox responses to humans in situations with different levels of interaction

between the experimenter and tested animal. Foxes were tested in their home cages, by an observer, in four steps:

Step A	Observer stands calmly near the closed cage but does not deliberately try to attract the animal's attention;
Step B	Observer opens the cage door, remains nearby but does not initiate any contact with the fox;
Step C	Observer attempts to touch the fox;
Step D	Observer closes the cage door, then stays calmly near the closed cage.

Videos demonstrating fox behavior in the standard test are available at the website: http://cbsu.tc.cornell.edu/ccgr/behaviour/index.html.

Each test step was 1 min long, the total length of the test was 4 min. No more than one test was given to any individual animal on the same day. All tests were performed by the same experimenter.

Fox behavior during the test was scored from the video records for 200 recordable observations (traits). Each trait was designed to be scored in a binary fashion (e.g. presence or absence). To evaluate the location of the fox in the cage, the space in each cage was partitioned into six zones. Zones 1-2 are located in the front of the cage (zone 2 is the closest to the experimenter), zones 5-6 are at the back of the cage, zones 3-4 are in the middle.

Two tests (test #1 and test #2) were scored for BCT, BCA, and F2 populations. Test #1 and test #2 were scored by two different observers, respectively. Only test #1 was scored for the tame, aggressive, and F1 populations.

Trait informativeness was evaluated from a combined data set that included the scores of foxes from tame (83 foxes), aggressive (80) and F1 (93) populations. Traits with less than 10% frequency of observations in the less-frequent category were removed from further consideration. The 98 traits retained (Supplementary Table I) represent all four test steps (A, B, C, and D) that were used to define fox behavioral phenotypes. In contrast to the previous study that concentrated on traits distinguishing between fox populations (Kukekova et al. 2008), the current study was focused on obtaining information for multiple dimensions of fox behavior and therefore all informative traits were retained for further analysis.

Averaged scores for tests #1 and #2 for BCT (293 foxes), BCA (202) and F2 (250) populations and test #1 scores for the tame (83), aggressive (80), and F1 (93) populations were combined in one data matrix (1003 foxes, in total) for principal component analysis (PCA). PCA was carried out: (1) for all test steps together (98 traits); (2) for each test step separately: Step A (24 traits), Step B (27 traits), Step C (30 traits), Step D (17 traits). Details of the principal component analysis are described below in "Principal component analysis" section. A summary of the first two PCs is presented in Fig. 1, Supplementary Table II and Supplementary Fig. 1.

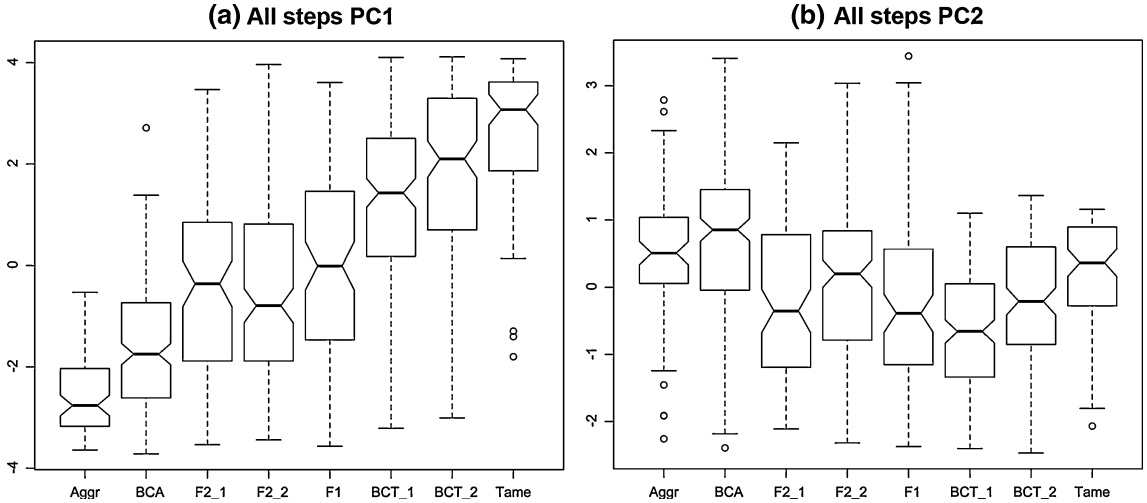

Fig. 1 Population distributions for the first two principal components of silver fox behavior. **a** Distributions for principal component 1 (PC1); **b** Distributions for PC2. Aggr = "aggressive" founder population; BCA = backcross-to-aggressive; F2_1 and F2_2 = two F2 populations (F1 × F1); F1 = F1 population ("tame" × "aggressive"); BCT_1 and BCT_2 = two backcross-to-tame populations; Tame = "tame" founder population. *Horizontal bars* within each box indicate the population median. Confidence intervals for the medians are shown as notches such that two distributions with non overlapping notches are significantly different ($P = 0.05$). The *bottom and top edges of the boxes* indicate the 25 and 75 percentiles. The *whiskers* indicate the range of data up to 1.5 times the interquartile range. Outliers are shown as *individual circles*. PC1 in the different populations exhibits a gradient, spanning the behavioral variation between that of the parental populations, that conforms to the expectation for a heritable component consistent with the overall contribution from "Tame" and "Aggressive" ancestry. This is clearly not the case for PC2

Genotyping fox pedigrees

In total, 385 SSR markers were used for genotyping fox pedigrees (Supplementary Table III). The marker set included 320 markers reported previously (Kukekova et al. 2004, 2007) and new microsatellite markers that were designed using the canine sequence assembly CanFam2 as described (Kukekova et al. 2007). New markers used for genotyping (developed after publishing the first meiotic linkage map of the fox) are listed in Supplementary Table IV. The majority of new microsatellite markers were designed to fill gaps over 15 cM that were present on the first meiotic linkage map.

BCT_1 pedigrees were genotyped with 340 markers distributed along all fox chromosomes except the Y chromosome; BCT_2 pedigrees were genotyped with 99 markers located on fox chromosomes 1, 3, 4, 5, 8, 12, 14, 15, and 16; BCA pedigrees were genotyped with 231 markers distributed along all chromosomes except the Y chromosome; F2 pedigrees were genotyped with 226 markers distributed along all autosomes.

Fox samples were genotyped at Cornell University and Marshfield Laboratories using a complementary approach. Detailed information about the origin of genotypes in each population is available in Supplementary Table III.

Fox samples were amplified with fluorescently labeled primers under the following conditions: an initial 2 min denaturation at 96°C; then 30 cycles of 96°C (20 s), 55°C (20 s), 72°C (20 s); and a final extension step at 72°C for 1 h. A subset of primers was genotyped using a 50°C annealing temperature (Supplementary Table III). PCRs were performed in 15 μl containing 0.3 pmol of each primer, 1.5 ng/μl of fox DNA and 1× GoTAQ (Promega, Madison, WI) master mix, or master mix containing 1× Invitrogen Taq Polymerase buffer, 1.5 mM $MgCl_2$, 0.2 mM of dNTP, and 0.5 units of Invitrogen Taq Polymerase. From 4 to 7 microsatellites were combined, post PCR, in multiplex sets and resolved on an ABI3730 Genetic Analyzer (PE Biosystems, Foster City, CA). F2 pedigrees were genotyped in part using PCR with multiplexed sets of markers (Supplementary Tables III, IV). PCR products were sized relative to an internal size standard using the ABI Genemapper 3.5 software package (PE Biosystems, Foster City, CA).

All genotypes were checked for data clarity, number of alleles, peak height and percentage of failed interrogations. Genotypes that passed the initial evaluation criteria were checked for Mendelian segregation using the prepare option of MultiMap (Matise et al. 1994) and manually corrected for errors.

Map construction

BCT_1, BCA, F2_1, F2_2 and three tame pedigrees were used for construction of a second generation fox meiotic linkage map. In total, 137 pedigrees with 635 offspring were used for map construction. Markers were assigned to linkage groups using the previous version of the fox map (Kukekova et al. 2007) together with the conserved synteny between the fox and dog genomes. The framework map and the subsequent LOD 2.0 map were generated using MultiMap (Green et al. 1990; Matise et al. 1994) as described previously (Kukekova et al. 2007). In total, 289 markers were uniquely placed on the LOD 2.0 map and a most likely location was defined for 92 markers (Supplementary Fig. 2). This LOD 2.0 map was used for association mapping.

Because GridQTL analysis requires all markers to be placed in unique positions, we generated an additional version of the fox map by saturating the LOD 2.0 map with previously unmapped markers without statistical support (LOD 0.0) (Supplementary Fig. 3).

Association mapping

Association between phenotype and SSR genotype was evaluated by the Pearson correlation between each individual's phenotypic value and SSR allele dosage count, for each SSR locus. Significance for single SSR alleles and thresholds for genome scans were established using permutation tests. The phenotypic values were permuted with respect to foxes and all SSR loci tested against the permuted values. This process was repeated 5,000 times to establish the null distribution for each SSR. A generalized extreme value distribution was fitted to this empirical null data using the gevFit function of the fExtreme package for R. The Kolmogorof-Smirnoff test of the R package (ks.test) was used to test the goodness of fit. Distributions with a ks.test *P*-value of 0.01 or less were considered poorly estimated and dropped from further analysis. The significance of rxy values were estimated using the cumulative probability function (pgev) and −log10 transformed for convenience (LOGP). For each permutation the maximum score across all SSR loci was recorded as the single genome-scan maximum. Genome-scan maximum values from 1,000 permutations were used to estimate the null distribution of a genome-wide scan. The 90, 95 and 99% percentiles of this distribution were used as the thresholds for genome-wide significance of 0.1, 0.05 and 0.01 respectively.

GridQTL mapping

QTL interval mapping used the web-available software GridQTL (Seaton et al. 2002, 2006). The algorithm BCF2 was used for mapping BCT and BCA pedigrees separately, and for mapping combined data sets that included BCT, BCA, and F2 pedigrees. The algorithm F2inbred was used for mapping F2 pedigrees separately from backcross

pedigrees. Chromosome-wide and experimental-wide significance thresholds were established by 1,000 permutations.

5,000 bootstraps were run on each population for each individual observation. For each bootstrap a random sample of foxes was selected, with replacement, and additive effects were estimated at each 1 cM point on VVU12. Populations were compared to each other using these bootstrap results. Significant differences in additive effects were estimated as the fraction of trials for which the difference in additive effects was reversed from the original results.

Principal component analysis

Principal component analysis was performed using the prcomp function in R (R Development Core Team 2006). Principal component analysis (Venables and Ripley 2002) defines independent factors (Eigenvectors or loadings) that describe a decreasing amount of the total variation. The interpretation of these Eigenvectors is important in exploring the biology underlying these patterns of variation. The magnitude of the loadings and their relative signs (correlations or inverse correlation) describe the influence of different traits on Principal Components defining aspects of behavior. Bootstrap analysis (Manly 1997) was used to place confidence intervals about the individual trait loadings for each Eigenvector as follows: (1) Run the principal component analysis (PCA) on the total set using the prcomp (Venables and Ripley 2002) function in the R program. This defines the "best" Eigenvectors (PCs), which will be used as standards for comparison to the bootstrap trials. (2) Randomly select, with replacement, a set of measurements equal in number to the total set. (3) Run PCA on this random set. (4) Find the PC which best matches each of the original PCs and record the loadings. (5) Repeat steps 2, 3 & 4, 5,000 times. The resulting lists of trait loadings for each principal component give an estimate of the confidence intervals about the "best" trait loadings from the original data. Patterns defined by the "best" PCs which are consistent with most of the data will reappear in most of the bootstrap trials. Important trait loadings should remain significant—i.e. confidence intervals should not include 0. Here we denote the significance of the loadings as the ratio of the "best" loading value to the standard deviation estimated from the bootstrap trials (i.e. The number of standard deviations from zero).

Results

Heritability of tame and aggressive behaviors

As described previously, principal component analysis (PCA) was used to analyze fox behavior (Kukekova et al. 2008). Videotaped behavioral observations from a number of steps in the investigator's approach to, and interaction with each fox were assembled into a principal component matrix used to assess behavior. PC1 had been shown to be consistent with the segregation of tame behavior in a backcross-to-tame population (Kukekova et al. 2008) and consequently was used as a quantitative measure of the segregation of tame/aggressive behavior. Subsequently, selected F1 foxes were mated to produce different segregating populations comprising: two backcrossed-to-tame (BCT_1 and BCT_2), one backcrossed-to-aggressive (BCA), and two intercrossed F2 populations (F2_1 and F2_2). Behavioral observations from foxes in all of these populations were used to construct a PC matrix that could be used to compare behavior between populations. PC1 and PC2 calculated using this combined matrix were found to account for 33 and 9% of the total behavioral variation, respectively. Figure 1 compares population distributions for PC1 and PC2. PC1 conforms to the expectation for a heritable component consistent with an expected genetic gradient spanning the behavioral variation between the parental populations. This was not the case with PC2, nor did any other PC (PC3, PC4, etc.) show segregating variation consistent with the axis of selection used to produce the parental populations.

Genetic mapping of behavioral variation

A genome wide association study (GWAS) using SSR markers on the initial backcross-to-tame population (BCT_1) indicated significant association of PC1 with markers on VVU12 at 21.3 and 23.0 cM on the meiotic map (Table 1). A suggestive association of PC2 with markers at the same position on VVU12 was observed. The GWAS of the subsequent crosses identified a suggestive

Table 1 Association of the first two principal components of silver fox behavior with markers on VVU12

Population	SSR Marker	Map position (cM)	GW Threshold	Trait
BCT_1	REN282I22	21.3	0.01	PC1
	REN01G01	23.0	0.01	PC1
	REN282I22	21.3	0.1	PC2
BCT_2	AHT137	22.2	0.1	PC1
F2	CM5.60	30.3	0.01	PC1

The first two principal components used in this analysis were defined using all test steps. A genome wide association study was performed for each data set (BCT_1, BCT_2, and the data set including both F2_1 and F2_2 populations) separately. "GW Threshold" indicates the genome wide threshold for significance that was exceeded by association mapping of principal components to the corresponding marker

association of PC1 with a similar position on VVU12 in segregants from a second backcross-to-tame population (BCT_2) and a highly significant association in F2 segregants at a position 10 cM away.

The data matrix used in the above analysis comprised all of the test steps (A, B, C & D—see Materials and methods) used for behavioral analysis. For further analyses we constructed PC matrices using separate data sets from each of the steps used in behavioral testing. Again, all of the populations were included in each matrix to facilitate comparison between populations. The results from these separate GWAS are presented in Table 2. Loci again were implicated on VVU12 for only PC1 and PC2. However, the significance of association was increased in particular steps (B and C) and significant associations were noted for the backcross-to-aggressive (BCA).

Microsatellite markers used in these GWAS often do not distinguish between alleles with the same size derived from different founding populations, and this results in decreased power to detect loci. To overcome this, taking advantage of the meiotic map of the fox, we undertook interval mapping of PC1 combining data from all steps and all informative populations. This confirmed the QTL on VVU12 (see Fig. 2), with a PC1-associated signal detected at a significance level exceeding the experiment wide threshold at $P < 0.05$ (Table 3).

Table 3 and Supplementary Fig. 4 compare interval mapping results for the combined test stages (A–D) with interval mapping using individual stages in different segregating popultions. We identified specific regions on VVU12 in BCT_1 and BCT_2 associated with PC1 and PC2 using the matrix with all stages included. Additionally, stages A and B were significant for PC1 (A.PC1, B.PC1) in these backcrosses-to-tame, as was stage C for PC2 (C.PC2). For the F2 population, stage C was highly significant for PC1 (C.PC1) and there was marginal significance for PC2 when all of the stages were combined. These data implicate a QTL on VVU12 in the genetics of the behavior selected during fox domestication.

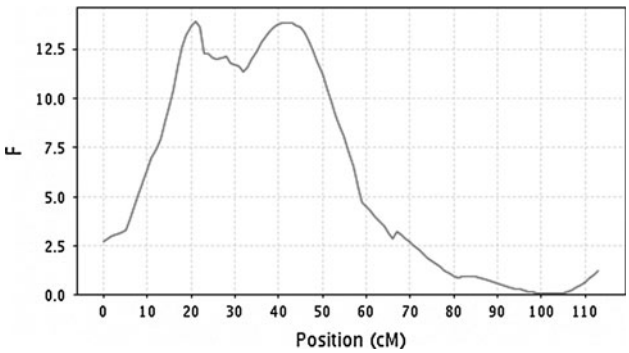

Fig. 2 Interval mapping of the first principal component of silver fox behavior. Interval mapping of PC1, using GridQTL software, was undertaken on a combined data set including all experimental silver fox populations. The *F* stat (*y*-axis) is graphed as a function of cM distance across the VVU12 chromosome. Interval mapping across all populations yields supports for a PC1-associated loci on VVU12, located broadly between 10 and 60 cM, that exceeds the threshold for genome wide association at a significance level $P < 0.05$ (Table 3), and confirms the mapping in individual populations (see Tables 1, 2, and 3)

Table 2 Association of behavioral components 1 and 2 for individual test steps with markers on VVU12

Population	Marker	Map position (cM)	GW Threshold	Trait
BCT_1	REN01G01	23.0	0.05	B.PC1
	CM35.11b	22.6	0.05	C.PC1
	CM35.13d	23.45	0.05	C.PC1
	REN282I22	21.3	0.05	C.PC2
	REN01G01	23.0	0.05	C.PC2
BCA	FH3393	4.9	0.01	D.PC1
	REN01G01	23.0	0.01	C.PC2
F2	CM5.41b	57.5	0.01	B.PC1
	CM5.41b	57.5	0.01	C.PC1
	CM5.60	30.3	0.01	C.PC1
	CM5.41	28.4	0.01	C.PC1

A genome wide association study was performed for each data set (BCT_1, BCA, and the data set including both F2_1 and F2_2 populations) separately. "GW Threshold" indicates the genome wide threshold for significance that was exceeded by association mapping of principal components to the corresponding marker. The test step is indicated by the first letter in the trait abbreviation (e.g. B.PC1 corresponds to the PC1 defined using behavioral observations from the test step B "Observer opens the cage door, remains nearby but does not initiate any contact with the fox")

The data in Tables 1, 2, 3, Fig. 2, and Supplementary Fig. 4 implicate a region on VVU12 between 10 and 60 cM. Comparative alignment of VVU12 against the dog genome (see Supplementary Fig. 5) indicates that conserved synteny of dog chromosome 5 with VVU12 begins at ~27 cM on the fox meiotic map. Part of this interval is orthologous to a region in the dog genome identified by vonHoldt et al. (2010), as demonstrating a signature for positive selection in the domestication of dogs from wolves.

Two striking aspects of the mapping data are: (1) the variation in the strength and location of the PC1 QTL signal with different stages in different populations and (2) the association of PC2 with VVU12.

Characteristics of behavioral traits comprising the tame/aggressive gestalt

PC1 is constructed from a matrix of 98 behavioral observations during a structured interaction with an investigator: In step A the investigator has approached the fox cage but

Table 3 Interval mapping of behavioral components 1 and 2 for individual test steps and all test steps combined in different segregating populations

PC	VVU12	F	LOD	Chromosome wide (0.01)	Experiment wide (0.05)	Experiment wide (0.01)
All experimental populations						
PC1	21 cM	13.9	3.0	10.2	12.3	15.1
BCT_1 and BCT_2						
PC1	39 cM	13.1	2.8	11.0	12.6	15.4
PC2	37 cM	**22.9**	4.8	9.7	12.5	15.0
A.PC1	39 cM	*9.4*	2.0	9.3	12.8	16.7
B.PC1	39 cM	**19.1**	4.0	10.5	13.1	17.1
C.PC2	33 cM	**17.9**	3.7	10.0	12.5	15.2
F2_1 and F2_2						
PC2	47 cM	*12.3*	2.6	11.3	13.4	17.4
C.PC1	47 cM	**22.7**	4.7	11.9	13.1	16.1

Interval mapping was performed separately for each of three data sets: (i) all experimental populations combined (BCT_1, BCT_2, BCA, F2_1, and F2_2), (ii) backcross-to-tame population (BCT_1 and BCT_2), (iii) F2 population (F2_1 and F2_2)

F statistics values in *italics* indicate that interval mapping results exceeded chromosome wide significance threshold at $P < 0.01$; *Underlined* *F* values indicate that interval mapping results exceeded experiment wide significance threshold at $P < 0.05$; *F* values in *bold* indicate that interval mapping results exceeded experiment wide significance threshold at $P < 0.01$. Interval mapping graphs are presented in Fig. 2 and Supplementary Fig. 4

the cage is closed; in step B the investigator is calmly standing next to the open cage; in step C the investigator is trying to touch the fox; in step D the investigator is calmly standing next to the closed cage. To analyze the genetic basis for fox behavior in greater detail, we used interval mapping to identify QTLs on VVU12 for individual behavioral traits.

Supplementary Table I presents observed frequencies of the 98 behavioral traits in experimental and parental fox populations. In general, the frequencies of traits associated with the tame behavior conform to the values expected on the basis of the genetic cross: 75% in the backcross-to-tame, 25% in the backcross-to-aggressive, and 50% in the F2 population. In the following examples (Table 4), individual traits that contribute to the PC1 gestalt are analyzed.

Figures 3, 4, 5 and 6 present interval mapping of 12 of the 98 traits used in evaluating the behavioral response of foxes to humans. The four figures represent quite different categories of behavior: Fig. 3 evaluates three physical positions of the ears, representing different responses to the investigator standing next to the open cage (Fig. 3b), or trying to touch the fox (Fig. 3a, c). Figure 4 analyzes three different behavioral responses to the presence of the investigator next to the open cage (Fig. 4a) or during the stage when the investigator is trying to touch the fox

Table 4 Frequency of trait observations in individual experimental populations

Figure	Trait	Trait description	BCT_1	BCT_2	BCA	F2
3a	C12	Tame ears	0.88	0.84	0.13	0.36
3b	B25	Pinned ears (aggr.)	0.07	0.05	0.60	0.26
3c	C35	Narrow ears directed back	0.33	0.22	0.18	0.29
4a	B15	Sniffing the front wall/door	0.64	0.75	0.42	0.51
4b	C6	Observer can first touch fox in zones 3-4	0.44	0.45	0.08	0.49
4c	C18	Fox holds observer's hand with its mouth	0.28	0.45	0.04	0.18
5a	C37	Aggressive sounds	0.09	0.07	0.80	0.47
5b	C30	Attack	0.04	0.02	0.53	0.23
5c	C33	Trying to bite	0.05	0.04	0.66	0.31
6a	B11	Comes into zones 1-2	0.69	0.81	0.36	0.56
6b	B2	Immediately moved back to zone 5 or 3-5-6	0.81	0.72	0.83	0.73
6c	D14	Sits in zone 2 looking at observer	0.57	0.52	0.09	0.22

The figure for which mapping results are displayed is indicated in the first column. The trait code and brief description is shown followed by the frequencies observed in each population. Frequencies of traits in the F2 population were calculated using both F2_1 and F2_2 data sets

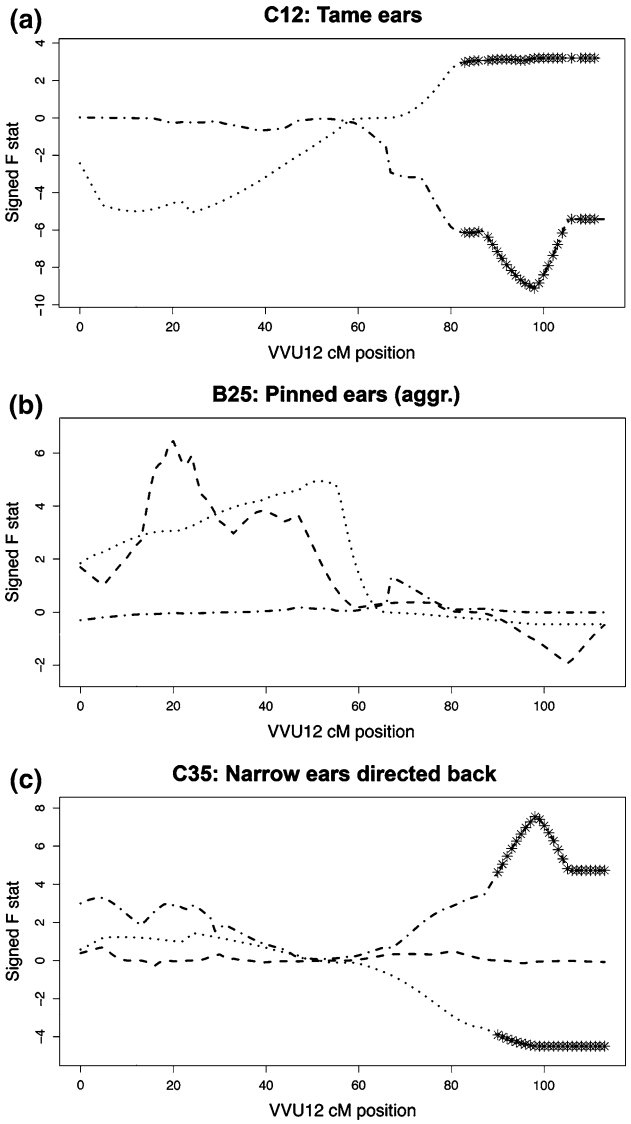

Fig. 3 Interval mapping of 3 fox behavorial traits defined by ear position, in 3 different segregating populations. For each trait, the signed *F* statistic (*y*-axis) from GridQTL is plotted as a function of cM distance across VVU12 (*x*-axis). The sign of the *F* statistic indicates the direction and parent-of-origin of the additive allele effect (i.e. positivity indicates that the allele originating from the tame population increases the frequency of the observed trait in the segregating population, and negativity indicates that the "tame" allele decreases the trait frequency). **a** Trait C12, "Tame ears", in populations BCT_1 and BCT_2; **b** Trait B25, "Pinned ears", in F2, BCT_1 and BCT_2; **c** Trait C35, "Narrow ears directed back", in F2, BCT_1 and BCT_2. BCT_1 = dotted line, BCT_2 = dot dash line, F2 = dashed line. In plots **a** and **c**, where BCT_1 and BCT_2 differ with significance $P < 0.001$, the plot lines are emphasized with stars. Traits C12 ("Tame ears", plot **a**) and C35 ("Narrow ears directed back", plot **c**) map in a complementary manner. In BCT_2 a QTL maps near 100 cM for which the "tame" allele decreases the frequency of observation of trait C12 (**a**) and increases the frequency of C35 (**c**). In BCT_1 this QTL has either no effect, or a small effect of opposite sign; and no QTL effect is evident in the F2 population. The difference in effect between BCT_1 and BCT_2 is highly significant. Trait B25 ("pinned ears", a behavior typical of foxes in the aggressive founder population) yields no support for a QTL near 100 cM in any of the 3 segregating populations, or anywhere on VVU12 in BCT_2, but does suggest a QTL in the 0–60 cM interval in the F2 and BCT_1 populations, with a tame allele having a positive effect on trait frequency

(Fig. 4b, c), all involving different tactile reactions: seeking to be touched or being touched by the investigator. Figure 5 involves aggressive actions when the investigator tries to touch the fox. Figure 6 involves three different distancing reactions of the fox to the presence of the investigator standing next to the open cage (Fig. 6a, b) or leaving the cage (Fig. 6c). These involve coming close to the investigator, retreating or not moving.

An observation common to all of these data is that interval mapping using different populations results in quite different outcomes: Thus the presence of the tame allele in the two different backcross-to-tame populations, or in the intercross (F2) often resulted in quite different effects (Figs. 3, 4, 5a, 6). Although some traits map to the same region of VVU12 in different backcross populations (Figs. 3a, c, 4b, 6c) others map to additional regions of VVU12 (Fig. 3a) or fail to map in one or the other population (e.g. Figs. 4c, 5b, c). Results involving segregation in intercross populations often supported trait mapping in one of the backcross populations (Fig. 5a, c), but often traits that mapped in backcross populations failed to map in the intercross populations (e.g. Figs. 3c, 6a). These effects help to explain why the strong heritability observed for PC1 in Fig. 1 did not translate into an equally definitive identification of QTL loci in different populations.

The different mapping characteristics of behavioral traits of a given type suggest different genotype/phenotype relationships. In Fig. 3, different ear positions are associated with different regions of VVU12 and associations vary between populations: Tame ears suggest that two loci located between 0 and 60 and between 60 and 120 cM are involved. Pinned ears, a characteristic of aggressive behavior, appear to involve only the region on VVU12 between 0 and 60 cM. Narrow ears, that indicate uncertainty and a fear response, involve both regions in one of the backcrosses, but the association is less pronounced in the other, and is not seen in intercross segregants. In Figs. 4 and 5a, different interactions between the fox and investigator are associated with different regions of VVU12. However, the association results differ between the backcross-to-tame populations. Note that a "tame" allele in the region between 0 and 60 cM is promoting the same behavioral phenotype in the backcross-to-tame and the backcross-to-aggressive population (Fig. 5). However, these two populations differ in the association of behavioral phenotypes with the more distal region of VVU12

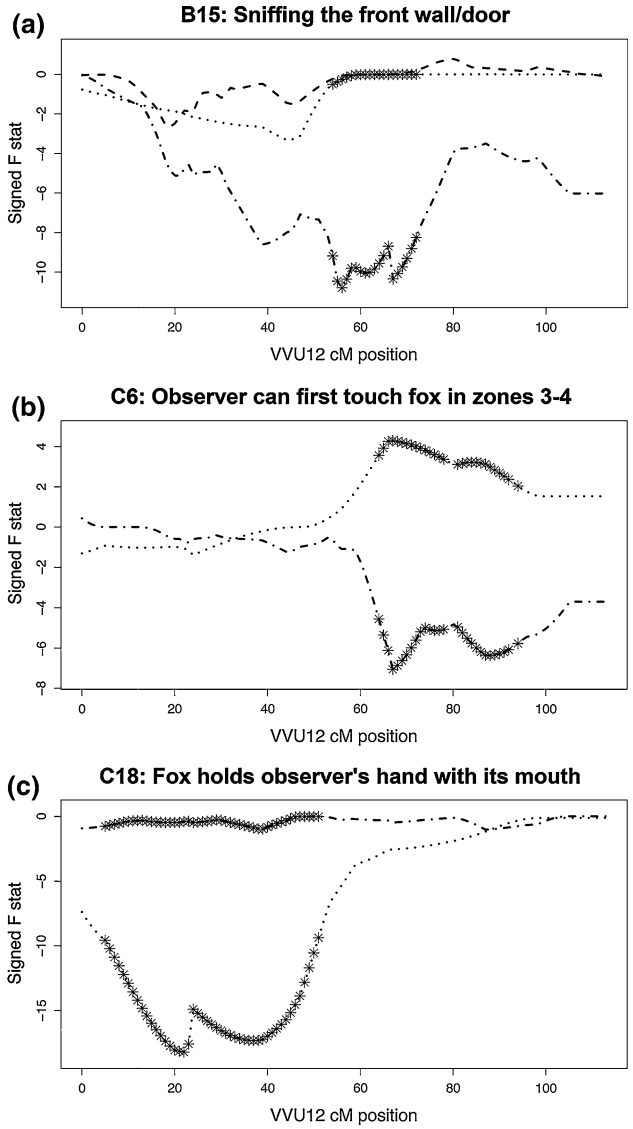

Fig. 4 Interval mapping of 3 "confrontational" fox behavorial traits, in different segregating populations. Plot formats and symbols as in Fig. 3. **a** Trait B15, "Sniffing the front wall/door [of cage]", in populations F2, BCT_1 and BCT_2; **b** Trait C6, "Observer can first touch fox in zones 3-4", in populations BCT_1 and BCT_2; **c** Trait C18, "Fox holds observer's hand with its mouth", in populations BCT_1 and BCT_2. Traits B15 and C6 support a QTL in the approximately 60–90 cM interval, for which the "tame" allele has negative effect (i.e. decreasing trait frequency) in BCT_2, but has no (B15) or a small positive effect (C6) in BCT_1—this difference is significant at $P < 0.001$. Trait C18 shows no support for a QTL anywhere on VVU12 in BCT_2, but in BCT_1 there is support for a QTL in the 0–60 cM interval, with the tame allele having negative effect, and this difference between BCT_2 and BCT_1 is also significant at $P < 0.001$

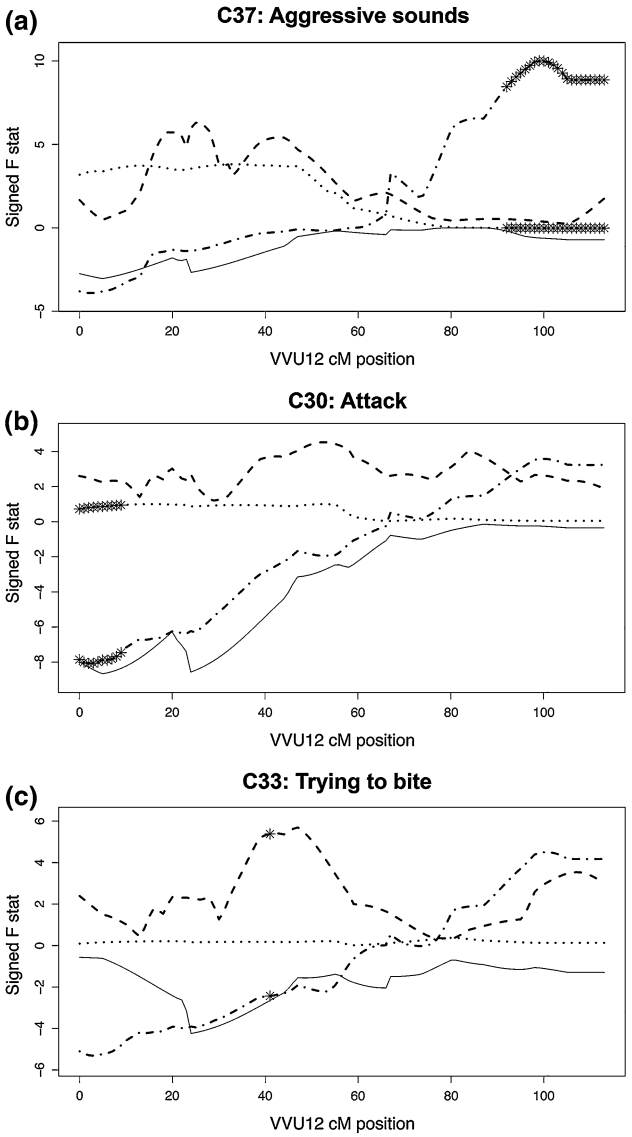

Fig. 5 Interval mapping of 3 "aggressive" fox behavioral traits, in segregating populations BCT_1, BCT_2, F2, and BCA. Continuous line = backcross-to-aggressive (BCA) population, otherwise plot formats and symbols as in Fig. 3. **a** Trait C37 ("Aggressive sounds"); **b** Trait C30 ("Attack"); **c** Trait C33 ("Trying to bite"). Trait C37 segregates in BCT_2 with a QTL at around 100 cM (**a**), with a "tame" allele having, counterintuitively, a positive effect (increasing trait frequency); no such effect is seen in the other populations, and the difference between BCT_2 and BCT_1 is significant at $P < 0.001$. Trait C30 is associated with a QTL in both the BCT_2 and BCA populations, in the 0–20 cM interval on VVU12, for which the "tame" allele has negative effect (reducing trait frequency), but this is not seen in BCT_1 or F2 populations. The difference between BCT_2 and BCT_1 for trait C30 is also significant at $P < 0.001$. There is no evidence for any QTL effect for trait C30 in the 60–100 cM interval in any of the populations. Trait C33 maps to the 0–60 cM region in F2 and BCT_2 populations but with the opposite effect. The same allele apparently has no effect in the BCT_1 population

(60–120 cM). Finally, in Fig. 6, two regions of VVU12 (0–60 and 60–120 cM) are associated with positional behaviors but segregation in one of the backcrosses only implicates the 0–60 cM region. In summary, the ability to associate different behaviors with regions of VVU12 differs from one segregating population to another and appears to depend on the genomic context of the segregating population.

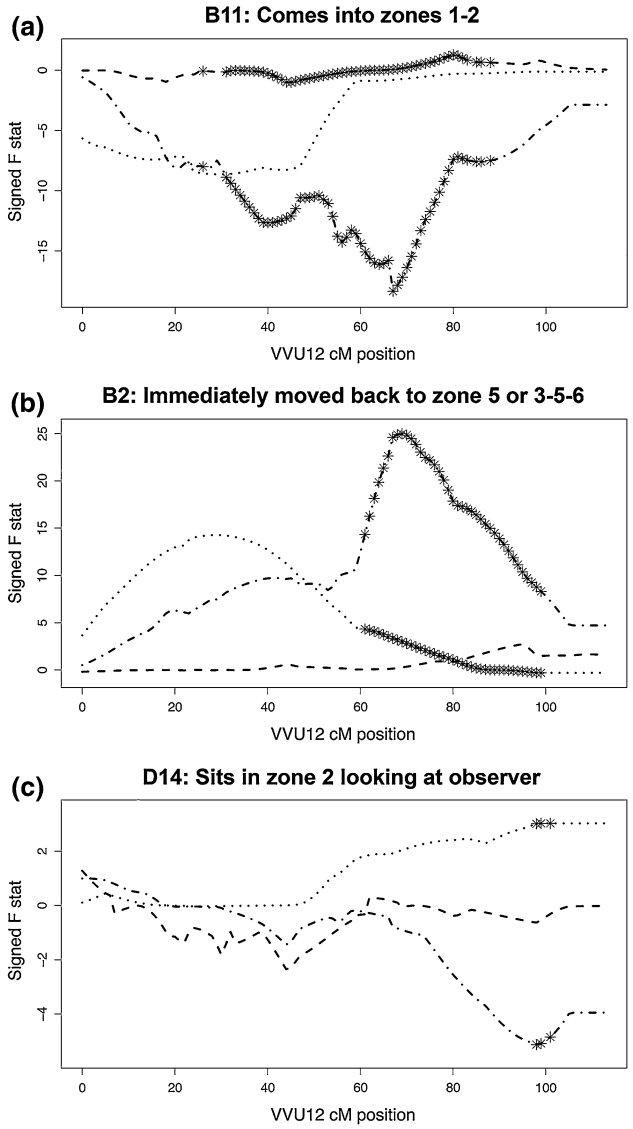

Fig. 6 Interval mapping of 3 "positional" fox behavorial traits, in segregating populations F2, BCT_1 and BCT_2. Plot formats and symbols as in Fig. 3. **a** Trait B11 ("[Fox] comes into zones 1-2"); **b** Trait B2 ("[Fox] immediately moved back to zone 5 or 3-5-6 "); **c** Trait D14 ("[Fox] sits in zone 2 looking at observer"). In the BCT_2 population a QTL mapping broadly to the 60–100 cM interval on VVU12 influences the observed frequency of all 3 traits. The direction of the effect is negative for traits B11 and D14 (i.e. the "tame" allele reduces the trait frequency), and positive for trait B2. The effect of this QTL is not apparent in the other populations

PC2 represents an active/passive behavioral gestalt

Genetic loci for PC2 also were identified on VVU12 using Step C (see mapping data above). Box graphs (Supplemental Fig. 1) comparing PC1 and PC2 using only step C observations of the different populations gave results similar to those for "all steps" (Fig. 1). We used step C trait loadings to compare the behaviors represented by PC1 and PC2. Table 5 contrasts the loadings for these two PCs. Whereas the loadings for PC1 show an inverse correlation between aggressive traits (aggressive sounds, attack, pinned ears, etc.) and tame traits (allows to touch nose, tame ears, allows to touch head, etc.), these traits are distributed in a quite different pattern in the loadings for PC2. In this PC, pinned ears and tame ears are correlated as are "comes to and sniffs observer's hand" and "follows the hand [aggressively]" as well as "tame noises" and "aggressive sounds", etc. The pattern of behavior that emerges for PC2 is an inverse correlation between passive and active behaviors. Thus, "fox rolls onto its side, inviting observer to touch its belly" and "attack" are active behaviors, while "observer can first touch fox in zones 5-6" and "Fox remains in zones 3-5-6" are passive behaviors. "Moved forward at least one zone during the step" and "spends more than 30 s in zones 3-4-5-6" are active and passive behaviors, respectively, but do not contribute much to distinguish foxes on the tame vs aggressive axis (PC1), since animals move forward both to greet (tame) or attack (aggressive).

Traits that contribute most to PC2, but not to PC1 also appear to map to VVU12. For example, "came into zones 1-2" (Fig. 6a) is an active trait; whereas "fox immediately moved back…" (Fig. 6b) is a passive trait. Other examples (Supplemental Fig. 6) include "Moved forward at least one zone", which had the highest "active" loading yet it contributed almost nothing to PC1 (see Table 5) or "observer can first touch fox in zones 5-6" that had the highest "passive" loading for PC2.

Discussion

Using a large number of specific behavioral characteristics (position in the cage, position of ears, position of tail, etc.) observed in the standard qualitative test for assessing silver fox behavior, we have developed a principal component matrix of orthogonal measures of behavior. The first component (PC1) provides a quantitative heritable phenotype that distinguishes between the aggressive and tame fox populations, and among populations and individuals derived from crosses between these two parental types (Fig. 1 and Supplementary Fig. 1). Thus PC1 scores for F1 foxes cluster about halfway between those characteristic of the two parental types, as do scores in the intercross F2 populations. Backcross-to-tame populations have PC1 scores segregating in a range midway between those in the F1 and tame populations, and scores for a backcross-to-aggressive population lie between those in the F1 and the aggressive populations. Using PC1 as a phenotype we have associated tame versus aggressive behavior with loci on VVU12, in a region orthologous to one recently identified in dogs and wolves as a locus for canine domestication

Table 5 Significances for each trait loading for the first two principal components of silver fox behavior at step C (C.PC1 and C.PC2)

Trait	Trait description	CPC1.SE	CPC2.SE
(a) C.PC1 (tame versusaggressive axis)			
C37	Aggressive sounds	−86	8
C34	Follows the hand (aggr.)	−84	14
C31	Attack alert	−83	12
C32	Pinned ears (aggr.)	−56	8
C36	Triangle ears directed back (aggr.)	−42	4
C30	Attack	−33	20
C33	Trying to bite	−33	12
C55	Leans on back or side walls in zones 5-6	−5	−14
C3	Fox is in zones 3-4-5-6 at the beginning of step C	−4	−24
C4	Spends more than 30 s in zones 3-4-5-6	−3	−33
C7	Observer can first touch fox in zones 5-6	−2	−41
C38	Fox remains only in zones 3-5-6	−1	−39
C50	Tail is up for at least for 3 s	−1	1
C39	Moved forward at least one zone during the step	3	29
C35	Narrow ears directed back	3	−26
C2	Fox is in zones 1-2-3-4 at the beginning of step C	6	24
C6	Observer can first touch fox in zones 3-4	18	4
C204	Tame sounds (combined)	18	10
C19	Comes into zone 2 at the end of step C	21	19
C25	Wagging tail	25	13
C18	Fox holds observer's hand with its mouth	26	14
C17	Fox rolls onto its side, inviting observer to touch its belly	27	20
C29	Comes to and sniffs observer's hand at the end of step C	32	14
C24	Loud breathing	36	20
C13	Fox allows observer to touch the rear part of its back	49	−18
C14	Fox allows observer to touch its back	73	−15
C8	Lies down during a contact for at least 5 s	88	0
C16	Fox allows observer to touch its head	105	−12
C12	Tame ears	107	7
C15	Fox allows observer to touch its nose	115	−10
(b) C. PC2 (active versus passive axis)			
C7	Observer can first touch fox in zones 5-6	−2	−41
C38	Fox remains only in zones 3-5-6	−1	−39
C4	Spends more than 30 s in zones 3-4-5-6	−3	−33
C35	Narrow ears directed back	3	−26
C3	Fox is in zones 3-4-5-6 at the beginning of step C	−4	−24
C13	Fox allows observer to touch the rear part of its back	49	−18
C14	Fox allows observer to touch its back	73	−15
C55	Leans on back or side walls in zones 5-6	−5	−14
C16	Fox allows observer to touch its head	105	−12
C15	Fox allows observer to touch its nose	115	−10
C8	Lies down during a contact for at least 5 s	88	0
C50	Tail is up for at least for 3 s	−1	1
C36	Triangle ears directed back (aggr.)	−42	4
C6	Observer can first touch fox in zones 3-4	18	4
C12	Tame ears	107	7
C32	Pinned ears (aggr.)	−56	8
C37	Aggressive sounds	−86	8

Table 5 continued

All observations from step C ("Observer attempts to touch the fox") are shown. The trait code and a brief description of the trait are in the first two columns. The significances for each trait loading for C.PC1 and C.PC2 are shown as the number of standard errors from zero (negative or positive) as established by bootstrap trials

Trait	Trait description	CPC1.SE	CPC2.SE
C204	Tame sounds (combined)	18	10
C31	Attack alert	−83	12
C33	Trying to bite	−33	12
C25	Wagging tail	25	13
C34	Follows the hand (aggr.)	−84	14
C29	Comes to and sniffs observer's hand at the end of step C	32	14
C18	Fox holds observer's hand with its mouth	26	14
C19	Comes into zone 2 at the end of step C	21	19
C24	Loud breathing	36	20
C17	Fox rolls onto its side, inviting observer to touch its belly	27	20
C30	Attack	−33	20
C2	Fox is in zones 1-2-3-4 at the beginning of step C	6	24
C39	Moved forward at least one zone during the step	3	29

(vonHoldt et al. 2010). Rat studies have identified two significant loci for domestication (Albert et al. 2009). However, these rat loci do not correspond to the fox locus on VVU12. Fox chromosome 12 corresponds to a fusion of three canine chromosomes: 5, 35 and 12 (Supplementary Fig. 5). Because canine SSR markers were used to construct the fox genetic map, the fox map can be compared and aligned to the dog genome. The conserved synteny between VVU12 and CFA5 starts around 27 cM on the fox map and continues to the telomere of both chromosomes. Thus, the independent domestication of the fox (farm-fox experiment, reviewed in Trut 1999, 2001; Trut et al. 2004, 2009) validates one of the major loci believed to be involved in the domestication of the dog.

A second behavioral component, PC2, corresponding to passive versus active behavior also maps to this chromosome. Although independent, by definition, the phenotypes measured by PC1 and PC2 are not entirely unrelated, in that activity can enhance differences in behavior that otherwise might be difficult to distinguish (e.g. an aggressive fox that attacks is more obviously aggressive than one that is passive; and a fox that greets the investigator and wags its tail is more obviously "tame" than one that is merely permissive). However, there are specific behavioral characteristics that contribute to the PC2 gestalt but not to PC1 and vice versa. Thus, in Supplementary Fig. 6 "fox moved forward" is a major activity characteristic whether aggressive or tame and "ability to touch a fox in zone 5 or 6" is indicative of great passivity in either a tame or an aggressive fox. In PC1, pinned ears and tame ears readily distinguish aggressive and tame foxes, but these two ear conformations have little to do with distinguishing contrasts between the active/passive gestalt of PC2.

It seems evident that PC2 can enhance the expression of PC1. That is, if an animal is aggressive, passive behavior will reduce the expression of that trait (animal is wary but lies still) whereas active behavior will enhance the expression (attack, or avoid the investigator). In backcross populations the distribution of behavior is skewed toward the extreme of the recurrent parent, reducing the contrast between tame and aggressive behaviors. Under these circumstances PC2 will increase that contrast. We would therefore expect that whereas these are distinct principal components in a matrix composed of all populations, PC1 and PC2 could be correlated in particular backcross populations. This is in fact the case for the backcross-to-tame populations in which PC1 and PC2 are correlated ($r = 0.75$–0.8). In contrast, in F2 populations where the behaviors are more normally distributed, this is not the case ($r = -0.06$). As a consequence of this relationship between PC1 and PC2, the mapping of PC2 to VVU12 needs to be regarded with some caution—it could be argued that this may simply reflect enhanced expression of PC1.

PC2 described in this study has parallels to the "shyness–boldness" factor proposed as a fundamental axis of behavioral variation in humans and other species (Wilson et al. 1994) and subsequently identified in studies of canine personality (Svartberg and Forkman 2002; Svartberg 2005; Saetre et al. 2006) and found to be related to performance level in working dogs (Svartberg 2002). The relationship between fox PC1 and PC2 indicates that passive/active behavior is not context independent and can be influenced by overall animal motivation (e.g. driven by PC1). These results suggest that this "shyness–boldness" factor should be considered with caution because animal motivation in performing certain tasks can influence the evaluation of this personality dimension.

Although the multiple character/trait groupings using principal component analysis has been very useful in defining behavior, GWAS using this phenotype has been very challenging, with different outcomes in different segregating populations. The data in Figs. 3, 4, 5 and 6

make it clear that the same loci may determine different trait outcomes in different populations. The overall frequency with which each trait outcome associated with "tameness" rather than "aggressiveness" is observed is broadly consistent with the percentage of the tame genome in each population: around 75% in the backcross-to-tame, 25% in the backcross-to-aggressive and 50% in the F2 segregating populations (see Supplementary Table I). It is not surprising, therefore, that we find differences in trait mapping (e.g. Figs. 3, 4, 5, 6) between the backcross-to-tame, the backcross-to-aggressive and the F2 populations. It is surprising, however, that we find differences between the two backcross-to-tame populations. Although the frequencies of individual trait phenotypes remain very similar between these two populations (see Table 4 and Supplementary Table I), the VVU12 mapping profiles (Figs. 3, 4, 5) are significantly different. This suggests that the loci on VVU12 may be expressed differently in different genomic contexts, depending on alleles elsewhere in the genome. This finding is consistent with the results from rats (Albert et al. 2009) that demonstrated the existence of a five locus epistatic network influencing tameness. The size of our F2 populations is currently too small to evaluate epistatic interaction between loci on VVU12 and other less significant loci identified in fox pedigrees.

Tameness as a defining characteristic of domesticated animals comprises a very complex phenotype. In the informative fox populations described herein we have dissected apart multiple distinct traits that in different combinations produce a tame gestalt. Perhaps the most obvious example of this multiplicity is the combination of passive/active (PC2) with tame/aggressive (PC1) behaviors, which interact to create an impression of greater or lesser affinity/acceptance or aversion/fear in the interaction between fox and human.

Hare (Hare et al. 2002, 2005) has shown that domesticated dogs can detect human intent (theory of mind), an ability which can provide the much needed mutual trust that is required for domestication. In the absence of language, communication must rely heavily on signals conveyed by motions or body language. These signals are provided by actions such as the positioning of the animal relative to the human interrogator, expressions of body language (ear position, tail wagging), and vocalizations. The suite of traits that combine to provide variations of the tame gestalt appear from the farm-fox experiment to be quite complex. It seems reasonable that a similar path was followed in the wolf/dog transition. The homology of loci described on dog CFA 5 and fox VVU12 attests to this similarity.

The data presented here will be important for studies of behavioral traits in mixed data sets that are often used in behavioral analysis of dogs and other species including humans.

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