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Comparative mtDNA sequence (control region, ATPase 6 and NADH-1) divergence in *Hucho taimen* (Pallas) across four Siberian river basins

E. Froufe*†, S. Alekseyev‡, I. Knizhin§ and S. Weiss¶**

*CIBIO/UP, Campus Agrário de Vairão, 4480-661, Vairão, Portugal, †Faculdade de Ciências, Universidade do Porto, Praça Gomes Teixeira, 4000-002 Porto, Portugal, ‡N. K. Kol'tsov Institute of Developmental Biology (IDB), Russian Academy of Sciences, 117334, Moscow, Vavilova 26, Russia, §Irkutsk State University, ul K. Marksa 1, Irkutsk, 664003, Russia and ¶Karl-Franzens University Graz, Institute of Zoology, Universitätsplatz 2, A-8010 Graz, Austria

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Hucho taimen from eight populations spanning four drainage basins (Amur, Lena, Enisei and Khatanga) were analysed for nucleotide sequence variation across three mitochondrial genes (ATP6, NADH-1 and control region). Samples of *H. hucho*, *Brachymystax lenok* (sharp-snouted and blunt-snouted forms) and *Parahucho perryi* were also included for comparison. Nucleotide variation across a total of 1826 base pairs in *H. taimen* revealed shared haplotypes between the Amur and Lena basins, further supporting a previous hypothesis of late to post-Pleistocene hydrological exchange between these now disjunct basins. In contrast to an earlier study using the control region alone, clear phylogeographic structure was seen at a large geographic scale, reflected by two phylogroups, one corresponding to the Amur and Lena basins, and the other to the Enisei and Khatanga basins. Comparative rates of divergence revealed considerably faster and less heterogeneous substitution rates for the two coding genes, especially at interspecific levels compared to the mtDNA control region.

Key words: *Brachymystax lenok*; *Hucho hucho*; *Parahucho perryi*; phylogeography; Salmonidae; Siberia

INTRODUCTION

Taimen *Hucho taimen* (Pallas), along with its sister taxon the Danube salmon or huchen *Hucho hucho* (L.) (designated as subspecies *H. h. taimen* and *H. h. hucho* by Hensel & Holčík, 1982) are among the largest and most endangered members of the Salmonidae (Holčík *et al.*, 1988). *Hucho taimen* occurs in north-eastern Europe (upper Volga and Pechora River basins) and in Siberia from the Ob River in the west to the Yana River in the north-east and to the Amur River basin in the south-east (Berg, 1948; Holčík *et al.*, 1988). Little molecular genetic

**Author to whom correspondence should be addressed. Tel.: +43 316 380 5599; fax: +43 316 9785; email: steven.weiss@uni-graz.at

data exist for the species, apart from single DNA sequences, RFLP, or allozyme data included in interspecfic phylogenetic studies (Osinov, 1991; Osinov & Lebedev, 2000; Shed'ko & Ginatulina, 1993; Phillips *et al*., 1995; Shed'ko *et al*., 1996; Phillips & Oakley, 1997; Crespi & Fulton, 2004), and a comparative phylogeographic study using seven sequences of the mitochondrial control region (CR) from east Siberian populations (Froufe *et al*., 2003*a*). In the last study, no variation in 600 bp of the CR was found, although samples came from three different river basins (Lena, Amur and Enisei). This contrasts to substantial mtDNA sequence variation found across the same region in *Brachymystax lenok* (Pallas), and the deep (> 4%) fixed divergence seen among major river basins within the genus *Thymallus* (Koskinen *et al*., 2002; Froufe *et al*., 2003*a*, *b*). For taimen, Froufe *et al*. (2003*a*) concluded that substantial cross-basin movements must have occurred during one of the most recent glacial periods, but also that various biological characteristics (long life span, small population size or metabolic rate differences) may also have contributed to the apparent reduction in interbasin nucleotide diversity compared to other salmonids in the same region.

The CR in salmonids, however, exhibits substantial evolutionary constraints, evidenced by a calibrated substitution rate for *Thymallus* of 1% per million years (Koskinen *et al*., 2002; Weiss *et al*., 2002), as well as a lower relative divergence rate compared to several salmonid genera than ATP6 (Froufe *et al*., 2005). In three of the four salmonid genera evaluated (*Thymallus*, *Salvelinus* and *Oncorhynchus*) ATP6 was shown to evolve considerably faster than the CR. This is congruent with the fact that several other coding segments (NADH subunits 1, 2, 5 and 6) have also been shown to evolve faster than the CR in salmonids (Hansen & Loeschcke, 1996; Apostolidis *et al*., 1997). Thus, the aims of the present study were to re-examine the phylogeographic structure of taimen in eastern Siberia, using additional samples as well as two coding genes (ATPase 6 and NADH1), which presumably evolved faster than the CR, and thus might show variation among the major river drainages.

MATERIAL AND METHODS

SAMPLING

Hucho taimen ($n = 17$) were collected by angling and gillnetting (a small fin clip was preserved in 96% ethanol) from eight populations belonging to four river basins (Table I and Fig 1). Outgroup taxa included two individuals of *B. lenok*, one sharp-snouted and one blunt-snouted (referred to as *B. tumensis* by Shed'ko, 2001), one *H. hucho* and two Sakhalin taimen *Parahucho perryi* (Brevoort). Sample locations are provided in Table I.

AMPLIFICATION AND SEQUENCING

Whole genomic DNA was extracted using a standard high-salt protocol (Sambrook *et al*., 1989). Three mtDNA genes, ATPase 6 (ATP6), NADH-1 (ND1) and CR were amplified using polymerase chain reaction (PCR). The ATP6 gene was amplified in all individuals, using the L8558 and H9208 primers described in Giuffra *et al*. (1994). The ND1 primers were designed from a *Salvelinus* sequence (GenBank accession number NC_000861), and were used to amplify all individuals (BlNDF- TAAG GTGG CAG AGCCCGGTA, BlNDR- TTGAACCCCTATTAGCCACGC). The CR (including a

TABLE I. Sample locations including major river basins, geographical co-ordinates and number of individuals for *Hucho taimen* and the three outgroups used in this study: *Brachymystax lenok*, *Hucho hucho* and *Parahucho perryi*

Population	Basin	*n*	Latitude (N)	Longitude (E)	Genbank CR	Genbank ATP6	Genbank NDH-1
1 Gramna Lake	Tiya → Baikal → Enisei	1	55°44′	109°06′	AY230447	AY862343	AY862365
2 Bol'shaya River	Baikal → Enisei	4	54°28′	109°29′	AY230448; AY862325	AY862344-AY862347	AY862366-AY862369
3 Varlamovka River	Enisei	1	62°23′	89°25′	AY862326	AY862348	AY862370
4 Kotuikan River	Khatanga → Laptev Sea	2	70°31′	103°52′	AY862327-AY862328	AY862349-AY862350	AY862371- AY862372
5 Ichikta River	Kirenga → Lena	2	57°15′	108°05′	AY862329-AY862330	AY862351-AY862352	AY862373- AY862374
6 Kalar River	Vitim → Lena	1	56°14′	119°32′	AY230449	AY862353	AY862375
7 Kuanda River	Vitim → Lena	2	56°31′	117°26′	AY862332-AY862333	AY862354-AY862355	AY862376- AY862377
8 Sukpai River	Khor → Amur	4	47°45′	137°18′	AY230450; AY862336-AY862337	AY862356-AY862359	AY862378-AY862381
Brachymystax lenok (sharp-snouted)	Anui → Amur	1	49°17′	137°55′	AY862339	AY862361	AY862383
Brachymystax lenok (blunt-snouted)	Dyanyshka → Lena	1	65°27′	126°56′	AY862340	AY862362	AY862384
Hucho hucho	Austria	1			AY862338	AY862360	AY862382
Parahucho perryi	Tumnin → Sea of Japan	2	49°22′	140°15′	AY862341-AY862342	AY862363-AY862364	AY862385- AY862386

FIG. 1. Sample locations for this study. See Table 1 for population codes and names.

partial segment of both flanking tRNA genes) was amplified in 11 individuals, using the LRBT-25 and LRBT-1195 primers described in Uiblein *et al.* (2001). The remaining CR sequences were taken from previously published research (Froufe *et al.*, 2003*a*; GenBank accession numbers in Table 1).

The PCR conditions (25 μl reactions) were as follows: each reaction contained 19 μl H_2O, 2·5 μl 10x Promega Buffer B, 0·5 μl 10 mM of each primer, 1·5 μl 25 mM $MgCl_2$, 0·5 μl 10 mM dNTP's, 0·1 μl Promega *Taq* DNA polymerase and 0·5 μl 100 ng per μl DNA template. The cycle parameters were as follows: initial denaturation at 94° C for 3 min, denaturation at 94° C (40 s), annealing at 53° C for ATP6 and 55° C for ND1 and CR (40 s) and extension at 72° C (40 s) repeated for 30 cycles. Amplified DNA templates were purified using the NucleoSpin Extract Kit (Machery-Nagel) and *c.* 100 ng of purified PCR product was used in cycle sequencing reactions following ABI PRISM BigDye Terminator protocols. The sequencing primers were the same as the PCR reactions except for the ND1 where two internal primers were designed and used (BLNDF1- GCACAAACTATTTCCTACGA; BLNDR1- CGTTGAAGGTTTGAAGTGTG). Sequences were visualized on either an ABI-3100 or ABI-310.

SEQUENCE ALIGNMENT AND ANALYSIS

The ATP6 and ND1 sequences were aligned by eye based on the amino acid sequences using the standard mtDNA code for vertebrates in the BioEdit programme (Hall, 1999). The *H. taimen* CR sequences were aligned with the outgroup sequences using the Clustal X programme (Aladdin Systems, Heidelberg, Germany) with default gap costs. The alignment was further optimized by eye. The observed pairwise sequence divergence (uncorrected *p* distances) and the number of transitions and transversions were obtained using PAUP*4·0b10 (Swofford, 2002).

To assess the degree of saturation in each codon position of the ATP6 and ND1 sequences, the number of transitions and transversions was plotted against the uncorrected pairwise distances, for first, second and third positions. Variation among sequences in base pair composition was examined: base composition homogeneity was tested using a χ^2 test of base frequencies across taxa, examining each coding position separately for the ATP6 and ND1 data.

Unrooted networks were used for evaluating genetic relationships, since the genealogy of closely related haplotypes within basins, for the combined data set and for each gene alone was of particular interest. Networks were constructed with a 95% criterion (Templeton *et al*., 1992) and gaps counted as events (*i.e.* treated as a fifth state) using the TCS 1.13 computer programme (Clement *et al*., 2000). The divergence of haplotypes, or clades, whether within or beyond the limit of this parsimony criterion, is also given using the net nucleotide divergence (Da) between groups (Kimura two-parameter model) calculated in MEGA 2·01 (Kumar *et al*., 2001). This metric corresponds to between-group variation corrected for within-group variation in haplotypes (Nei, 1987). This same distance is used for pairwise divergence estimates between species.

RESULTS

The final alignment for all 22 individuals, including outgroup sequences included 663 bp for the ATP6 gene, 552 bp for the ND1 and 661 bp for the CR. The ND1 gene was 3 bp shorter for taimen and huchen. For the CR there were nine 1–2 bp indels in the interspecific alignment. Based on a χ^2 test of homogeneity, there were no significant differences in base frequencies across taxa for any gene. Plotting the number of substitutions against uncorrected *p*-distances revealed that neither transitions nor transversions were saturated in any of the gene regions, including analysis of the third codon positions alone for coding genes (unpubl. data). For coding genes there were no stop codons and within taimen there were no amino acid changes. The transition: transversion ratio was 7·0 for ATP6, 10·1 for ND1 and 8·0 for CR. The most variable gene segment was ND1 (113 variable sites; 102 parsimony informative) followed by ATP6 (107; 87) and CR (69; 51).

For taimen there were a total of four haplotypes for the ATP6 gene, seven for ND1, five for CR, and 11 considering all gene segments combined. The distribution of haplotypes among populations for all genes is shown in Table II. Pairwise sequence divergence was nearly identical for all gene segments (*c*. 0–1·3%). For all segments except CR, two divergent groups are evident with 1% (ATP6), 0·8% (ND1) or 0·7% (all genes) difference between them (Fig. 2). Net sequence divergence between species ranged from a minimum of 1·5% for taimen and huchen at either the ATP6 or ND1 gene, to a maximum of 16·3 % for *P. perryi* and *B. lenok* at the ND1 gene (Table III).

The two coding genes reveal very strong concordant phylogeographic structure as one of the divergent groups is found exclusively in the Enisei and

TABLE II. List of haplotypes and their frequencies for the three genes analysed (ATP6, ND1 and CR), across all eight populations sampled ($n = 17$ taimen individuals). Note that Bol'shaya and Gramna populations are connected to Lake Baikal, which is part of the Enisei basin

	Populations (number of individuals)							
	Basin							
	Enisei			Khatanga	Lena			Amur
Genes and haplotypes	Gramna	Bol'shaya	Varlamovka	Kotuikan	Itchitkta	Kalar	Kuanda	Sukpai
ATP6								
H1	1	4						
H2			1	2				
H3								3
H4					2	1	2	1
ND1								
H1				2				
H2		4						
H3	1							
H4			1					
H5							1	3
H6					1		1	
H7					1	1		1

TABLE II. Continued

	Populations (number of individuals) Basin							
	Enisei			Khatanga	Lena			Amur
Genes and haplotypes	Gramna	Bol'shaya	Varlamovka	Kotuikan	Itchitkta	Kalar	Kuanda	Sukpai
CR								
H1		1	1	2				
H2					1			
H3							2	2
H4	1	3				1		2
H5					1			
All genes								
H1	1							
H2		3						
H3		1						
H4				2				
H5			1					
H6								1
H7								2
H8					1			
H9							2	
H10					1			
H11						1		1

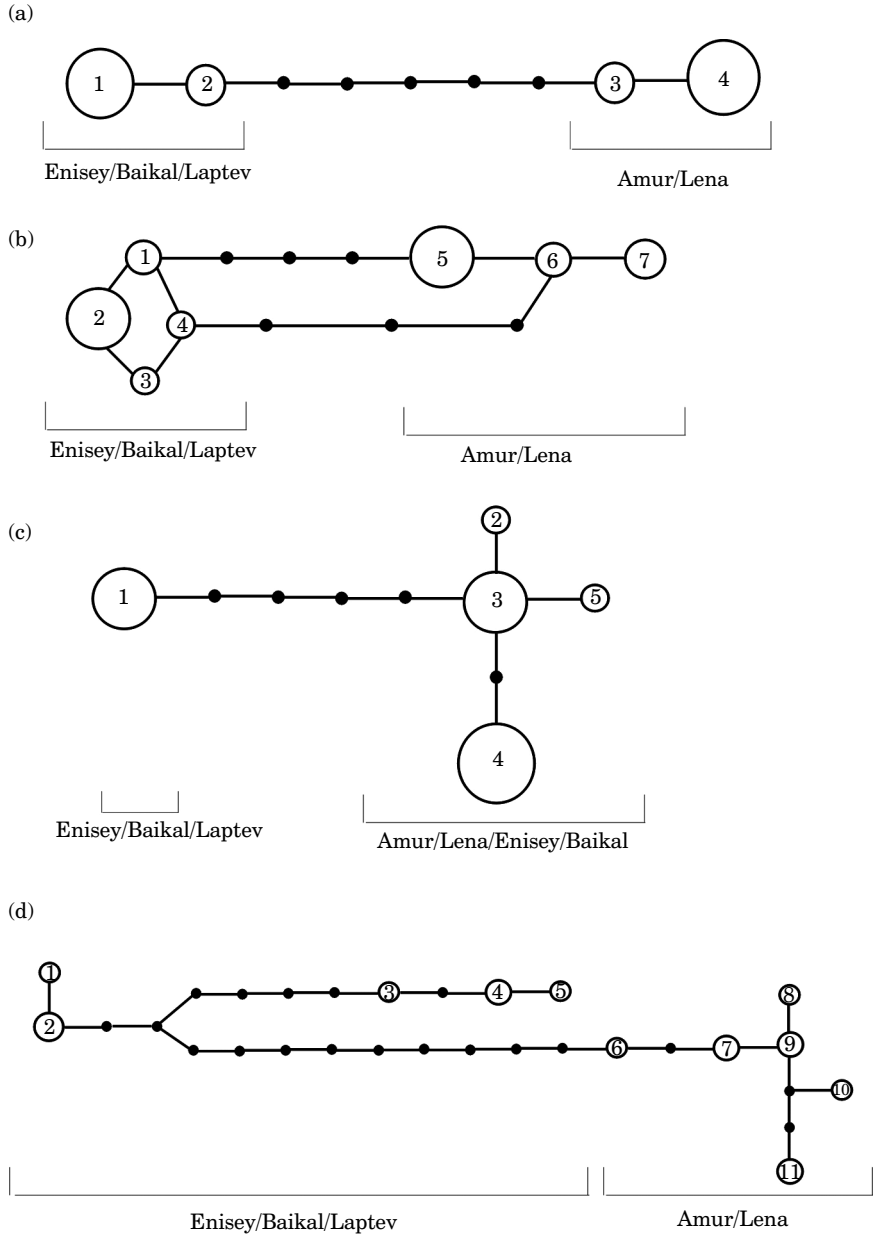

FIG. 2. Parsimony networks of *H. taimen* haplotypes for (a) ATPase 6 (663 bp), (b) NADH-1 (552 bp), (c) control region (611 bp) and (d) combined sequence data. For all networks circle size is proportional to the observed haplotype frequencies. ●, unobserved haplotypes.

Khatanga basins and the other in the Amur and Lena basins. For the ATP6 gene, the Enisei/Khatanga group contained one haplotype found in all individuals from Bol'shaya and Gramna populations (both tributaries to Lake Baikal), and another in both the Kotuikan and Varlamovka Rivers (Table II).

TABLE III. Net pairwise sequence divergence (Kimura 2-parameter) between the four taxa [*Hucho taimen, Hucho hucho, Brachymystax lenok* (both forms) and *Parahucho perryi*] derived from each gene data set separately as well as together. (a) ATP6 values in lower diagonal; ND1 values in upper diagonal. (b) CR values in lower diagonal; values for the combined analysis in upper diagonal

(a)	*H. taimen*	*H. hucho*	*B. lenok* (sharp)	*B. lenok* (blunt)	*P. perryi*	(b)	*H. taimen*	*H. hucho*	*B. lenok* (sharp)	*B. lenok* (blunt)	*P. perryi*
H. taimen		0·015	0·077	0·078	0·152			0·016	0·062	0·061	0·112
H. hucho	0·015		0·093	0·093	0·160		0·017		0·067	0·064	0·112
B. lenok (s)	0·064	0·072		0·057	0·158		0·040	0·031		0·032	0·111
B. lenok (b)	0·062	0·073	0·028		0·163		0·036	0·038	0·013		0·116
P. perryi	0·112	0·115	0·133	0·126			0·074	0·070	0·060	0·057	

For the ND1 gene, each of the four populations in the Enisei/Khatanga group contained a unique haplotype. For the Amur/Lena group, each of the coding genes revealed at least one shared haplotype between these two basins (Table II).

The geographic distribution of CR haplotypes was not as distinct as for the coding genes [Fig 2(c) and Table II]. The network contains three closely related haplotypes (H2, H3, H5) found exclusively in the Amur and Lena basins (two shared between these basins), resulting in a clade analogous to the Amur/Lena group based on the coding genes. The broadly distributed haplotype H4, is two mutations divergent from this group. A single haplotype (H1), five mutations divergent from H3, is fixed in the Enisei/Khatanga basins. With all three gene segments combined the two phylo-groups are also resolved [Fig 2(d) and Table II).

DISCUSSION

Previous assessment of the phylogeographic structure of *H. taimen* in eastern Siberia left a somewhat puzzling picture of genetic homogeneity across three major, currently disjunct basins (Froufe *et al*, 2003*a*). Despite the extremely small sample sizes presented, it was argued that increased sample sizes could not invalidate the conclusion that genetic diversity in taimen reveals an inter-basin shared ancestory more recent than that seen in other salmonids (*Thymallus* and *Brachymystax*) in the region (Froufe *et al*, 2003*a*). *Hucho taimen* is an increasingly rare and endangered species, and thus samples are not easily obtained. The current sampling of eight sites (across four basins) has more than doubled the number of individuals, and more importantly, has nearly tripled the number of nucleotides analysed (*c*. 1826). Thus, an overturn of previous conclusions was not expected, but the goal to increase phylogeographic resolution was met.

The analysis of the CR alone revealed five haplotypes, including two new haplotypes from the same sites previously sampled (Bol'shaya and Sukpai). Some large-scale phylogeographic structure is revealed as the newly described haplotypes all have limited geographic ranges. The presence of a common haplotype (H4) across the three largest basins (Amur, Lena, Enisei), however, still supports hydrological exchange at some time in the past.

The addition of two more genes revealed further phylogeographic structure, especially for the ND1 gene, which displayed population specific haplotypes in the Enisei and Khatanga basins. Both of these genes, however, also showed shared haplotypes between the Amur and Lena basins, concordant with inter-basin shared ancestory of CR haplotypes. River capture events between the upper Amur and Lena basins are relatively well-documented with paleogeological data (Pavlovskii 1939; Korzhuev 1956, 1979; Ganeshin, 1972), which is further supported with molecular data on *Thymallus* (Froufe *et al*., 2005) and *Brachymystax* (Froufe *et al*., 2003*a*).

At a larger geographic scale, the Amur and upper Lena basins appear to have remained disjunct from the Enisei and Khatanga basins over the most recent relevant paleohydrological events (*i.e*. events associated with the advance and retreat of the last glaciation). This appears true not only for *Hucho*, but *Brachymystax* and *Thymallus* as well, a biological observation that is not

concordant with one of the hypotheses of Grosswald (1998, 1999), that during the Pleistocene the Lena and Enisei basins were repeatedly connected. According to Mats *et al.* (2001) Baikal discharged into the Lena River from *c.* 2 to 0·5 million years ago. After that the waters of Lake Baikal 'broke out' forming a connection with the Enisei first *via* the Irkut (a tributary of the Angara River) (0·5–0·06 million years ago), and later *via* the Angara proper, a paleohydrological event reflected in the molecular architecture of *Thymallus* in the region (Koskinen *et al.*, 2002). The present data on *Hucho* are concordant with the hypothesis that the Lena and Enisei basins have been separated for at least 0·5 million years (*c.* three glacial cycles), and, as for *Thymallus*, reveal no molecular signature of a Lena basin lineage in the Baikal basin.

Despite the higher phylogeographic resolution of the coding genes compared to the CR, overall divergence values for each gene segment are nearly identical (1·2 to 1·3%). The maximum pairwise divergence seen at the CR, however, does not correspond to phylo-groups *per se* but rather the divergence between two haplotypes, the widespread CR-H4 and the newly described CR-H1, found in three localities in the Enisei/Khatanga region. This haplotype is defined by four apomorphic substitutions within 30 bp, three occurring at adjacent sites, raising the suspicion that they may in fact be correlated (*i.e.* represent a single mutational event). Thus, the divergences involving this haplotype are likely to be less linear with time than divergences among haplotypes of the coding genes. This observation is in concordance with the known substitution rate heterogeneity in the vertebrate CR (Hasegawa *et al.*, 1993), a problem that has motivated the development of correction factors such as the shape parameter (α) of the gamma (Γ) distribution.

An example of this in salmonids is seen in the analysis of the genus *Thymallus*, where the estimated α for the CR was less than half (0·573) than that estimated for the ATP6 gene (1·147) (Froufe *et al.*, 2005), reflecting a relatively drastic change in the substitution rate heterogeneity (the same sequence model and nearly the same estimate of invariable sites was used for both genes). For intraspecific studies, this heterogeneity, especially over short divergence times, can confound phylogeographic inferences.

Irrespective of substitution rate heterogeneity, the generally slower rate of substitution for the CR is more clearly seen in the interspecific comparisons. Maximum divergences between *P. perryi* and either *Hucho* or *Brachymystax* taxon range from 11·2 to 16·3% for the coding genes but only 5·7 to 7·0% for the CR (Table III). In fact, all pairwise comparisons reveal considerably higher divergences for the coding genes, except for the *H. hucho* x *H. taimen* where the CR is 1·7% divergent compared to 1·5% for either coding gene.

This analysis is the first demonstration of phylogeographic structure in the genus *Hucho*. The distribution of mtDNA variation in taimen is further shown to be somewhat concordant with other salmonids in the region, and supports some paleohydrological hypotheses concerning the interface of the Amur and Lena catchment basins. The genetic data on *Hucho*, however, is sparse, and the current understanding of phylogeography in Siberian fishes lags considerably behind the information base in North America and Europe.

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